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MASTER OF SCIENCE

Compounds Modulating Calcium and Motility in Human Spermatozoa

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# Compounds Modulating Calcium and Motility in Human Spermatozoa

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MSc by Research

Human Reproductive Biology

University of Dundee

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**Declaration**

I declare that the content of this project report is my own work and has not previously been submitted for any other assessment. The report is written in my own words and conforms to the University of Dundee's Policy on plagiarism and academic dishonesty. Unless otherwise indicated, I have consulted all of the references cited in this report.

Signature:

Date:

## **List of Abbreviations**

AC: Adenylyl cyclase  
 ACU: Assisted conception unit  
 AI: Artificial insemination  
 ALH: Amplitude of lateral head displacement  
 ANOVA: Analysis of variance  
 AR: Acrosome reaction  
 ART: Assisted reproductive technology  
 ATP: Adenosine triphosphate  
 BCF: Beat-cross frequency (Hz)  
 BSA: Bovine serum albumin  
 $\text{Ca}^{2+}$ : Calcium ions  
 $[\text{Ca}^{2+}]_i$ : Intracellular calcium concentration  
 cAMP: Cyclic adenosine monophosphate  
 CASA: Computer-aided sperm analysis  
 CICR: Calcium induced calcium release  
 CM: Capacitating medium  
 $\text{CO}_2$ : Carbon dioxide  
 DDU: Drug discovery unit  
 DFI: DNA fragmentation index  
 DGC: Density gradient centrifugation  
 DMSO: Dimethyl sulfoxide  
 DNA: Deoxyribonucleic acid  
 DOR: Delta opioid receptor  
 dUTP: deoxyuridine-triphosphatase  
 FCM: Flow cytometry  
 FISH: Fluorescence in-situ hybridisation  
 FITC: Fluorescein isothiocyanate  
 FSC: Forward scatter  
 GPCR: G-protein coupled receptor  
 GTP: Guanosine-5'-triphosphate  
 HA: Hyaluronic acid  
 $\text{HCO}_3^-$ : Bicarbonate ions  
 HFEA: Human Fertilisation and embryology authority



HOS: Hypo-osmotic swelling  
 HSA: Human serum albumin  
 HTS: High throughput screening  
 ICSI: Intracytoplasmic sperm injection  
 IUI: Intrauterine insemination  
 IVF: In-vitro fertilisation  
 $K^+$ : Potassium  
 KOR: Kappa opioid receptor  
 LIN: Linearity (VSL/VCL)  
 MAO: Monoamine oxidase  
 MAR: mixed antiglobulin reaction  
 MC: Methyl cellulose  
 mRNA: messenger ribonucleic acid  
 MOR: Mu opioid receptor  
 $Na^+$ : Sodium  
 $NaHCO_3$ : Sodium bicarbonate  
 NaOH: Sodium hydroxide  
 NCB: Non-capacitating buffer  
 NCM: Non- capacitating HEPES-buffered medium  
 NO: Nitric oxide  
 NOS: Nitric oxide synthase  
 PCT: Post coital test  
 PDE: Phosphodiesterase  
 $pH_i$ : Intracellular pH  
 PKA: Protein kinase A  
 PN: Pronuclei  
 PSA: *Pisum sativum* (pea) agglutinin  
 PTX: Pentoxifylline  
 QMPT: Quantitative cervical mucus penetration test  
 rcf: Relative centrifugal force  
 ROS: Reactive oxygen species  
 sAC: Soluble adenylyate cyclase  
 SBTI: Soy-bean trypsin inhibitor  
 SEM: Standard error of the mean  
 SMPT: Sperm mucus penetration test

SSC: Side scatter

STR: Straightness (VSL/VAP)

TdT: Terminal deoxynucleotidyl transferase

TESE: Testicular sperm extraction

TM: Transmembrane

TMC: Total motile count

TTP: Time to pregnancy

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

tmACs: transmembrane adenylate cyclases

UN: United Nations

UoD: University of Dundee

VAP: Average path velocity

VCL: Curvilinear velocity

VSL: Straight-line (rectilinear) velocity

WHO: World Health Organization

ZP: Zona pellucida

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## **Project Summary**

Infertility is a significant global problem, with ART cycles increasing every year. Male factor infertility is the single most common cause of infertility in couples, and as there are no therapeutic targets for males, the only treatment option is ART, which is expensive and invasive with no guarantee of success. The development of drugs to target asthenozoospermic (low sperm motility) males is a valuable area of research, with clinical potential to improve the take home baby rate.

Previous work using a Flex Station HTS assay and calcium as a surrogate for motility, identified 10 hit compounds from the UoD DDU Selleck MIH prescribable library that induced an increase in  $[Ca^{2+}]_i$  in human spermatozoa *in-vitro*. This thesis investigated the effect of this  $[Ca^{2+}]_i$  using techniques including CASA, Kremer tests and Flow cytometry to study compound effects on human spermatozoa motility and function. Some drugs demonstrated positive effects on spermatozoa motility characteristics, without an increase in function or AR. Furthermore, the drug compounds elicited similar positive effects on spermatozoa from subfertile patients, illustrating their clinical potential.

# **CHAPTER 1**

## **General Introduction**

## **Chapter 1: Thesis Introduction**

### **1.1 Infertility**

‘Infertility’ has been termed as a disease by the World Health Organisation (WHO) (Zegers-Hochschild et al., 2009), affecting 1 in 7 heterosexual couples worldwide (HFEA, 2013) in their reproductive life cycles. It is defined as the inability of a couple to achieve a clinical pregnancy after 12 months of unprotected sexual intercourse (Zegers-Hochschild et al., 2009), and is estimated to affect 3.5 million people in the UK alone (HFEA, 2012). There are many different causes of infertility, with physiological defects in men being the most common (Hull et al., 1985), either in isolation or in combination with female factor infertility. Other causes include female factors, for example anovulation, fallopian tube damage, and endometriosis (HFEA, 2013). Also, some cases are due to a problem with both the male and the female. In a significant proportion of couples – 25%, no cause for infertility can be found (NICE, 2013). Some of these cases could be explained by spermatozoa dysfunction, where semen analysis parameters appear normal but spermatozoa do not function correctly.

### **1.2 Male Factor Infertility**

The WHO undertook a large, international study of fertile males, with the aim of presenting their semen characteristics and providing reference intervals (Cooper et al., 2010). Fertile male controls were defined as couples who had a Time to Pregnancy (TTP) < 12 months and these were cross referenced with i) males from the general population with unknown fertility, ii) normozoospermic males (according to the then current WHO 1999 references) and iii) fertile males with unknown TTP. One sided lower reference limits – the fifth centile for fertile males - were generated; the average semen volume is 1.5ml, total spermatozoa number 39 million per ejaculate, concentration of  $15 \times 10^6/\text{ml}$ , 32% progressive motility, 40% total (progressive and non-



progressive) motility and 4% morphologically normal spermatozoa. With reference to these values, male factor subfertility is defined as a spermatozoa concentration of  $\leq 15 \times 10^6/\text{ml}$ , progressive motility of  $\leq 32\%$ , and total motility  $\leq 40\%$  (Cooper et al, 2010). Spermatozoa dysfunction is a significant global issue and its prevalence is continuing to negatively affect Artificial Reproductive Technology (ART) (Barratt et al., 2011; Tomlinson et al., 2013).

There are many different diagnostic semen analysis abnormalities, including; aspermia - absence of semen, azoospermia - absence of spermatozoa in the ejaculate, oligozoospermia - low spermatozoa concentration ( $\leq 15 \times 10^6/\text{ml}$ ), asthenozoospermia - poor spermatozoa motility ( $\leq 40\%$  total motility), and teratozoospermia - increased morphologically abnormal spermatozoa ( $\leq 30\%$  morphologically normal sperm) (WHO, 1992). These spermatozoa defects can occur as individual abnormalities in isolation or in combination.

Asthenozoospermia is a common cause of male infertility, reportedly affecting approximately 19% of infertile males (Curi et al., 2003). Motility is arguably the most significant clinical abnormality. British Fertility Society guidelines for practice report the chance of pregnancy increases with increasing numbers of motile spermatozoa in the ejaculate (Tomlinson et al., 2013). Larsen et al., (2000) indicated that cumulative conception rate correlates with increased percentage motility in the spermatozoa sample, specifically high velocity grade a motile spermatozoa; velocity  $>25 \mu\text{m}/\text{sec}$  as defined by the WHO (WHO, 1999). 430 couples were studied over 6 menstrual cycles and a relationship between increased concentration of motile spermatozoa and increased velocity independently compared with increased pregnancy rates was observed.

### 1.3 Artificial Reproductive Technology

The only treatment options for infertile couples is ART - except in rare circumstances, for example hypogonadotropic hypogonadism; dysfunction in the pituitary gland or hypothalamus affecting the production of sex hormones (Krausz, 2011). Since the first *in-vitro* fertilisation (IVF) baby born in 1978, it is estimated that around 5 million babies have been born worldwide through assisted conception. With increasing numbers of couples going through assisted conception (HFEA, 2013) it is clearly perplexing that the take home baby rate following ART remains 28% (on average), a figure that has stayed largely unchanged for many years (ART; National Summary Report, 2012). Spermatozoa dysfunction is a significant global issue, and currently there is no drug that a man can take, or that can be added to his spermatozoa *in-vitro*, to increase its function (Tardiff et al., 2014). The possibility to target asthenozoospermia with drug treatments to improve motility, functional ability and potentially ART success rates is attractive and clinically relevant. The overarching aim of improving the clinical techniques currently in place in the field of ART is to increase the take home baby rate for infertile couples.

ART is invasive and expensive, with no guarantee of success. There are three main ART treatment options available depending on the severity of spermatozoa dysfunction. Intrauterine insemination (IUI) is used to treat mild male subfertility, IVF is used in cases of moderate male subfertility and intracytoplasmic sperm injection (ICSI) in cases of severe male subfertility (Tournaye, 2012).

IUI is the simplest, least invasive and least expensive technique and it can be performed without expensive infrastructure (Cohlen, 2005). IUI requires a good spermatozoa concentration and motility. Cells are placed into the female reproductive tract using a catheter, therefore spermatozoa bypass the cervical mucus barrier and the cell density at

the site of fertilisation is increased (Ombelet, 2005). Opening up IUI to a greater patient population is important for developing countries who lack ART technology and infrastructure, and so IVF/ICSI are not readily available or affordable, and for developed countries where ART costs exclude many from treatment. But controversy remains over the effectiveness of this treatment when compared with IVF and ICSI (Ombelet, 2005; Pashayan et al., 2006). Reported pregnancy rates of IUI are much lower; between 10% and 18% per cycle (Stone et al., 1999; Kaplan et al., 2002; Cohen et al., 2005). Pashayan et al., (2006) reported an average live birth rate of 7% per cycle of stimulated IUI and suggested that IUI is not a cost effective treatment option as its failure rate is high, and it would be better clinical, and economic practise to direct couples straight to IVF or ICSI cycles. Targeting IUI spermatozoa samples with drugs which improve spermatozoa progressive motility would better enable cells to swim through the female reproductive tract, reach and fertilise the oocyte therefore potentially increasing the success rates of this treatment option.

IVF also requires a good spermatozoa concentration, motility and functional ability. Cells are incubated with the cumulus-oocyte complexes in a dish for a period of time to allow fertilisation. Fertilised oocytes are cultured for up to 5 days, to allow selection of the best 1 or 2 embryos to replace into the uterine cavity. Despite the highly medicalised approach to achieving a pregnancy, IVF still allows for some element of spermatozoa selection as cells must have the ability to penetrate the cumulus cells surrounding the oocyte, bind to the zona pellucida (ZP) and activate/fertilise the oocyte, therefore must have physiologically normal functions. Drug treatments which induce HA (hyperactivated) motility – a high amplitude, asymmetrical beating of the spermatozoa flagellum which aids in penetration of the cumulus cells and ZP of the oocyte – and spermatozoa functional ability would potentially increase the fertilisation rates of IVF cycles.

For males with severe spermatozoa dysfunction or very low spermatozoa concentration or motility, the recommended treatment option would be ICSI, the most expensive ART treatment, requiring skilled embryologists who have undergone extensive training. ICSI allows for no biological selection as a single spermatozoa is injected into each oocyte using an injection needle. The lack of physiological sperm selection raises concerns that cells with damaged or fragmented DNA may be chosen and injected into the oocyte. However, ICSI removes the element of chance involved in IUI and IVF cycles, as sperm will penetrate the oocyte as it is injected directly into it.

Currently the most common fertilisation technique is ICSI, accounting for around two thirds of all treatments worldwide and conventional IVF stands at around one third, although both treatment outcomes are comparable (Sullivan et al, 2013). In the UK, the most recent figures indicate that 52.6% of fresh treatment cycles in 2013 involved ICSI, and of these ICSI cycles, 53% were due to male factor infertility (HFEA 2013). IUI cycles have a lower success rate. The cumulative live birth rate was 13.6% after 3 IUI cycles inseminating <1 million motile cells, however it significantly increased to 22.4% when inseminating >1 million motile cells (Ombelet et al., 1997). The potential improvement of IUI cycle outcome would make it more attractive for use worldwide, particularly in countries where IVF and ICSI cannot be offered due to lack of infrastructure and specialists. Discovery of new drug therapies that can be administered to semen or prepared spermatozoa to increase motility or functional ability is an area of great potential and possibility, particularly as it is plausible that this would have substantial effects on the outcome of ART. IUI or IVF allocated spermatozoa samples would be targets for these motility enhancing compounds as they have the potential to increase fertilisation rates after use in these cycles.

#### 1.4 Diagnosis of Male Infertility

The diagnosis of male subfertility is predominantly done through manual semen analysis – visualising the sample down a microscope and manually counting concentration, motility and morphology of spermatozoa - using the parameters and methods defined by the WHO laboratory manual for the examination and processing of human spermatozoa (fifth edition). Semen analysis provides an estimate of relative fertility potential, and therefore has clinical utility, but carries limited clinical value for diagnosing infertility (Tomlinson et al., 1999; Vasan, 2011) in couples with unexplained subfertility. Only in the most extreme cases; males with azoospermia, can absolute values be provided. Semen analysis is used globally to assess male fertility as there is no other technique that has been developed that is thought to be more favourable, however it has its limitations. There is inherent variability between semen samples from individual men (WHO, 1992; Tomlinson et al., 1999), therefore the analysis of one semen sample from an individual is not representative of their overall fertility due to the heterogeneity within and between ejaculates (MacLeod & Irvine, 1995). Inter-observer variability in the andrology laboratory may provide ambiguous results. Also, the interval between production and analysis may vary between samples provided within and between clinics, therefore effecting the outcome of semen analysis.

By using CASA, - a computer software program attached to an external microscope which identifies the spermatozoa head and analyses movement characteristics and parameters such as velocity, track direction and concentration (Mortimer, 2000) - to estimate spermatozoa movement parameters improves the objectivity, precision, and reproducibility of the values (Krause, 1995) in comparison to measuring motility parameters manually. CASA also captures motility characteristics such as spermatozoa velocity and track direction. Specific spermatozoa motility variables such as progressive motility, average path velocity (VAP), linearity (LIN) and curvilinear velocity (VCL)

may be seen as prognostic indicators for the fertilisation potential of a spermatozoa sample (Joshi et al., 1996).

Given the limitations with current semen analysis, testing spermatozoa function determines their *in-vivo* potential, whether they have the biologic capacity to undergo the necessary functions required to reach and fertilise the oocyte, and ultimately the potential to result in live births (Vasan, 2011). Spermatozoa function assays are superior methods of testing fertilisation potential. Penetration of mucus is an effective test of motility and function, and more discriminating than semen analysis (Glazener & Hull, 1987). Kremer developed a method to determine the ability of spermatozoa to penetrate cervical mucus using capillary tubes (Kremer, 1965). *In-vivo*, spermatozoa are deposited into the anterior vagina at coitus where they rapidly make contact with visco-elastic substances such as cervical mucus, and enter the cervix where sperm selection begins (Suarez and Pacey, 2006). Immotile and morphologically abnormal spermatozoa are unable to pass through cervical mucus and are broken down by the immune cells (Rath et al., 2008), however cells of good motility and function are able to penetrate and swim through the viscous cervical mucus and move up the female reproductive tract. The Kremer test mirrors this physiological process and can determine the functional abilities of human spermatozoa and allows for a better evaluation of the prognostic value of these cells *in-vivo* (Aitken et al., 1985). Although this test is robust and has been found to have many advantages, such as being a reasonable predictor of fertility if spermatozoa are able to penetrate (Fjallbrant, 1968), it is not used in clinical practise. In terms of ART, this physiological process is bypassed, spermatozoa do not have to penetrate viscous media *in-vitro* or during an IUI cycle, however the functions required to penetrate the viscous media are similar to those required to penetrate and fertilise the oocyte, such as HA movement of spermatozoa.

There are other spermatozoa function tests, some are used clinically and others in research. Examples of such tests include acrosome reaction (AR) tests. The AR, which occurs at the point of fusion of the spermatozoa and oocyte, is characterised by the exocytosis of the acrosomal content and the release of hybrid vesicles formed by patches of the outer acrosomal membrane and the plasma membrane. It is required for binding and penetration of the zona pellucida (ZP) and subsequent fertilisation of the oocyte (Sagare-Patil et al., 2012). AR tests can determine if spermatozoa have prematurely undergone the reaction, indicating their fertilisation potential (spermatozoa are thought to be unable to fertilise after they have undergone the AR). Most of these tests are based on the binding of fluorescent acrosome specific probes in fixed cells (Cross & Meizel, 1989).

DNA damage/DFI tests; infertile male spermatozoa indicate increased DNA fragmentation (Irvine et al., 2000; Zini et al., 2001; Saleh et al., 2002), and this altered DNA integrity can lead to reduced ART results (Sun et al., 1997; Borini et al., 2006). A technique widely used, which detects DNA integrity is the terminal deoxynucleotidyl transferase [TdT]-mediated deoxyuridine triphosphate (dUTP) nick end labelling (TUNEL) assay. DNA fragmentation is assessed through the labelling with dUTP of the double- and single-stranded DNA breaks present in spermatozoa (Sailer et al., 1995). The comet assay (single-cell gel electrophoresis) is another way of assessing DNA damage by measuring DNA breaks in eukaryotic cells (Collins, 2004). A clinical method of assessing DNA fragmentation is a technique known as Halosperm, an improved sperm chromatin dispersion test (Anifandis et al., 2015).

Origio HBA slides; the ability of spermatozoa to bind to hyaluronic acid (HA) is a biochemical marker of spermatozoa maturity, morphology, reduced chromosomal abnormalities and DNA integrity (Yagci et al., 2010). Hyaluronan binding assay (HBA) slides are used in clinical practice and predict fertilisation ability of spermatozoa in IVF

cycles and distinguish spermatozoa samples suitable for IVF or ICSI (Breznik et al., 2013).

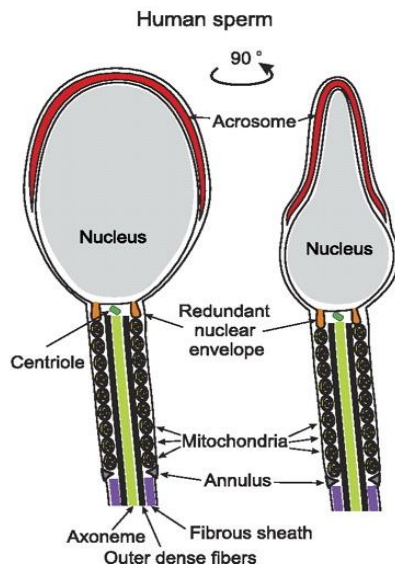
Zona free hamster oocyte penetration assay; Yanagamachi et al., (1976) developed a spermatozoa function test measuring the ability of spermatozoa to undergo capacitation (a spermatozoa maturation process that occurs in the female reproductive tract and is essential for spermatozoa fertilisation potential), fuse with the oocyte membrane and decondense the spermatozoa head resulting in the formation of the male pronucleus (Hwang & Lamb, 2013).

Acrosin assay; acrosin, a spermatozoa acrosomal serine proteinase, is involved in the AR, binding of spermatozoa to the ZP and is important for fertilisation (Kennedy et al., 1989). Assays determine spermatozoa samples acrosin levels, indicating cells fertilisation potential.

HOS test; when spermatozoa with intact semi-permeable membranes are placed in hypo-osmotic conditions, water enters the cell causing it to swell. Non-viable cells do not have an intact plasma membrane (Vasan, 2011) and so do not swell.



## 1.5 Sperm Physiology



**Figure 1.1: Physiology of Human Spermatozoa.**

The nucleus of the sperm cell is located in the head (which is overlaid by the acrosome) and takes up most of this space. Sperm cells contain very little cytosol. The centriole and the redundant nuclear envelope are located at the base of the head. The axoneme is surrounded by outer dense fibres in the midpiece and the principle piece of the spermatozoa. The outer dense fibres are surrounded by mitochondria in the midpiece, but by the fibrous sheath in the principle piece. The axoneme runs along all the flagella and is surrounded by mitochondria and outer dense fibres in the midpiece, and by outer dense fibres and fibrous sheath in the principle piece. The end piece contains only the axoneme (Darszon et al., 2011).

### 1.5.1 Capacitation

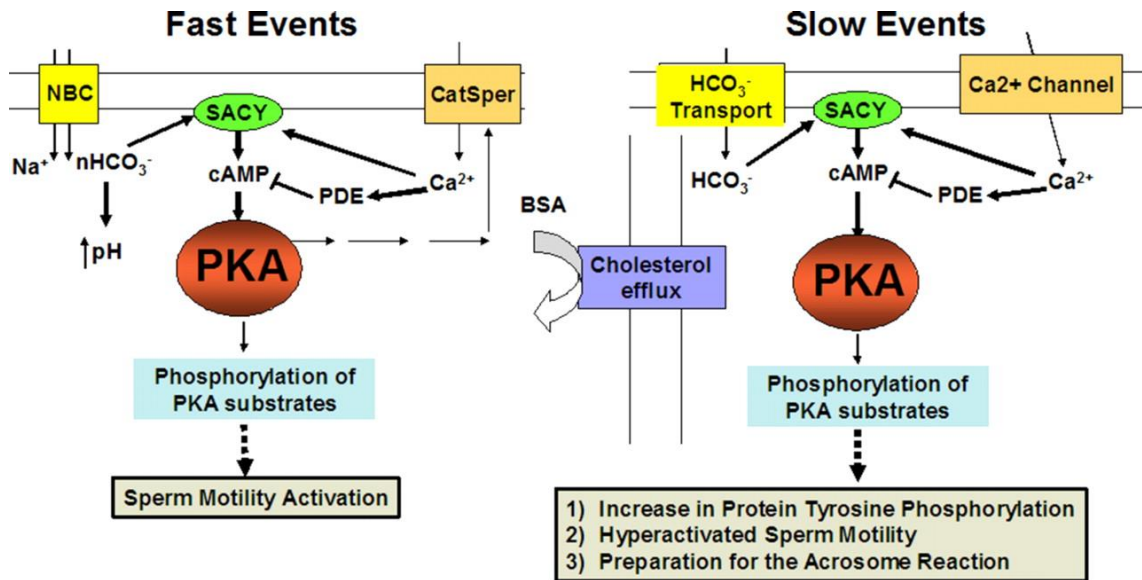
When spermatozoa are first ejaculated they are unable to fertilise an oocyte, and must undergo a process in the female genital tract, known as capacitation (Austin, 1951; Chang, 1951). Only after capacitation can mammalian spermatozoa acquire motility, which is initiated by changes in environmental conditions such as ionic concentrations or osmotic stimulation (Morisawa, 1994). Increase in protein phosphorylation (Uner and Sakkas, 2003; Visconti et al., 2011) through calcium signalling plays a central role in controlling activities that are vital for spermatozoa function, including motility, hyperactivation (HA), chemotaxis and the AR (Publicover et al., 2007; Darszon et al., 2011) (Figure 1.2). Early capacitation events and spermatozoa motility are also controlled by bicarbonate ( $\text{HCO}_3^-$ ), nitric oxide (NO), adenylate cyclase (sAC and tmAC), cAMP, and protein kinase A (PKA) pathways (Herrero et al., 2000; Lefievre et al., 2002; Salicioni et al., 2007; Visconti, 2009). *In-vitro*, capacitation can be initiated in spermatozoa by using media containing  $\text{Ca}^{2+}$ , serum albumin,  $\text{HCO}_3^-$  (Osheroff et al.,

1999) and metabolic substrates like glucose (Williams & Ford, 2001) and pyruvate (Hereng et al., 2011) which aid in the production of ATP.

There are many biochemical and molecular changes during capacitation. cAMP is thought to be a key player in the induction of HA - the high amplitude, asymmetrical beating of the spermatozoa tail, characterised by an increase in VCL and amplitude of lateral head displacement (ALH), and a decrease in the linearity of the cells swimming direction (Mortimer et al, 1998) - and acts through the activation of PKA, inducing tyrosine phosphorylation. Studies have shown that mice missing the sperm-specific PKA catalytic subunit alpha 2 (C alpha 2) are sterile and their spermatozoa do not HA (Nolan et al., 2004; Morgan et al., 2008). cAMP levels must be tightly regulated, both temporally and spatially, by AC (that synthesises cAMP from ATP (Lefievre et al., 2002)), and cAMP phosphodiesterases (Huston et al., 2006; Lynch et al., 2006). In vertebrates cAMP is synthesised by two types of AC; a ubiquitous family of transmembrane adenylyl cyclases (tmACs) with 9 members (Adcy 1 – 9) and sAC encoded by a single gene (Adcy10 aka SACY or sAC) (Buck et al., 1999; Jaiswal & Conti, 2003; Geng et al., 2005). tmAC is regulated by forskolin and heterotrimeric G proteins (Wertheimer et al., 2013), and plays a pivotal role in the regulation of the initial spermatozoa progressive motility as the cells enter and move through the female reproductive tract. It is involved in the basic activation of flagellar activity (Shiba and Inaba, 2014) and generates an increase in cAMP, inducing cells forward progressive movement (Dey et al., 2014).

sAC is also required for spermatozoa motility and capacitation (Hess et al., 2005; Xie et al., 2006) and is present in the spermatozoa flagellum (Wertheimer et al., 2013). sAC is stimulated by  $\text{HCO}_3^-$  and  $\text{Ca}^{2+}$  ions in the female reproductive tract (Chen et al., 2000; Jaiswal & Conti, 2003; Litvin et al., 2003), increasing flagellar beat and modification of membrane lipid architecture and organisation (Gadella et al., 2008), a prerequisite for

HA (Wennemuth et al., 2003; Esposito et al., 2004; Hess et al., 2005; Xie et al., 2006) through the increase in cAMP and activation of the PKA pathway inducing tyrosine phosphorylation (Figure 1.2).



**Figure 1.2:** Molecular basis of fast and slow events associated with sperm capacitation. (*Fast Events*) As soon as sperm are in contact with an isotonic solution containing  $\text{HCO}_3^-$  and  $\text{Ca}^{2+}$ , a vigorous flagellar movement is observed. This process requires an increase in PKA activity which is mediated by a  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$  coordinated stimulation of soluble adenylyl cyclase SACY.  $\text{HCO}_3^-$  is transported across the cell membrane by a  $\text{Na}^+/\text{HCO}_3^-$  cotransporter (NBC) and  $\text{Ca}^{2+}$  are transported by a sperm-specific  $\text{Ca}^{2+}$  channel (CatSper). (*Slow Events*) After capacitation, sperm acquire the ability to fertilize. The fertilization capacity is preceded by the preparation to undergo the exocytotic AR and the induction of hyperactivated motility. This is correlated with an increase in tyrosine phosphorylation, downstream of PKA stimulation; however, opposite to the fast processes, the increase in tyrosine phosphorylation also depends on the presence of cholesterol acceptors in the capacitation medium (Visconti, 2009).

Albumin, a cholesterol acceptor, has been shown to extract cholesterol out of the spermatozoa membrane changing fluidity - a  $\text{HCO}_3^-$  dependent process (Figure 1.2) (Osheroff et al., 1999; Visconti et al., 1999). The removal of cholesterol and other sterols (de-capacitating factors) from the plasma membrane produces a more fluid environment that aids in spermatozoa fertilisation abilities (Ikawa et al., 2010). This cholesterol release from human spermatozoa is also associated with an increase in cAMP and subsequently, protein tyrosine phosphorylation and capacitation (Osheroff et al., 1999).

NO also regulates human spermatozoa capacitation and protein tyrosine phosphorylation through the modulation of the cAMP and PKA pathway. The enzyme NOS is activated under capacitating conditions (Herrero et al., 2000).

### 1.6 Calcium and CatSper

Signals from the female reproductive tract and the oocyte-cumulus complex regulate spermatozoa motility HA and AR, thus fertilisation ability (Olson et al., 2011). HA is critical for the spermatozoa to ascend the female reproductive tract and fertilise the oocyte. This specific type of movement of the spermatozoa is required for migration through the highly viscous cervical mucus, and penetration of oocyte cumulus cells and the ZP (Suarez et al., 1991; Suarez and Dai, 1992; Stauss et al., 1995; Ren et al., 2001; Carlson et al., 2003; Quill et al., 2003; Ooi et al., 2014). Many males with dysfunctional spermatozoa have a reduced percentage of HA in their sample which is a major factor in their subfertility (Peedicayil et al., 1997; Munire et al., 2004). Clinical studies indicate that a reduction in the percentage of HA spermatozoa correlates with a reduction in *in-vitro* fertilisation rates (Sukcharoen et al., 1995). The lack of high amplitude movement of the spermatozoa will mean there is difficulty in penetrating the cumulus cells surrounding the oocyte and subsequently penetration of the ZP (Ooi et al., 2014). These functions are aided by the increased VCL and ALH during HA (Munire et al., 2004).

$\text{Ca}^{2+}$  is a major regulator of the initiation and maintenance of HA and the AR in mammalian spermatozoa. Calcium permeable channels exist in the plasma membrane of spermatozoa, and open to initiate signalling, allowing the flow of ions down their electrochemical gradients (Costello et al., 2009). There are two main calcium regulatory mechanisms in spermatozoa; entry of calcium via pH-dependent CatSper channels in the plasma membrane of the flagellar principle piece (Carlson et al., 2003), and through

mobilisation of stored calcium in the neck/midpiece region (Ho & Suarez, 2001, 2003; Marquez et al., 2007; Costello et al., 2009).

CatSper is a pH-sensitive voltage gated calcium channel exclusively expressed in the testis and in spermatozoa (Quill et al., 2001). As spermatozoa enter the female reproductive tract there is a change in ion concentration, which results in the hyperpolarisation of the spermatozoa plasma membrane (Demarco et al., 2003). It is thought that this may be partially due to an enhanced  $K^+$  permeability as a result of a decrease in inhibitory modulation of  $K^+$  channels (Demarco et al., 2003). Also, an increase in  $[Ca^{2+}]_i$  ion concentration also plays a role in membrane hyperpolarization (Lopez-Gonzalez et al., 2014). Previous studies using bioinformatics and gene-targeted transgenic mouse strategies have indicated that spermatozoa have a sperm-specific cation channel – CatSper – which is found to be crucial for HA. CatSper is a calcium channel composed of four pore-forming subunits; CatSper 1-4 (Ren et al., 2001; Quill et al., 2001) and three additional auxiliary subunits, CatSper beta, gamma and delta (Wang et al., 2009). CatSper defects have been studied in the spermatozoa of mutant mice and compared with wild type. CatSper 1-4 gene targeted transgenic mice all showed the same phenotype; male factor infertility, inability to HA and a decrease in motile spermatozoa over time, but the deletion does not affect spermatogenesis or initial motility after ejaculation. This would suggest that all of the pore-forming CatSper genes are essential for functional channels (Ren et al., 2001; Quill et al., 2003; Jin et al., 2007; Qi et al., 2007). Ren et al., (2001) illustrated that CatSper channels are necessary to allow spermatozoa to penetrate the ZP because CatSper 1-null mice were unable to fertilise ZP-intact oocytes but were able to fertilise ZP-free oocytes.

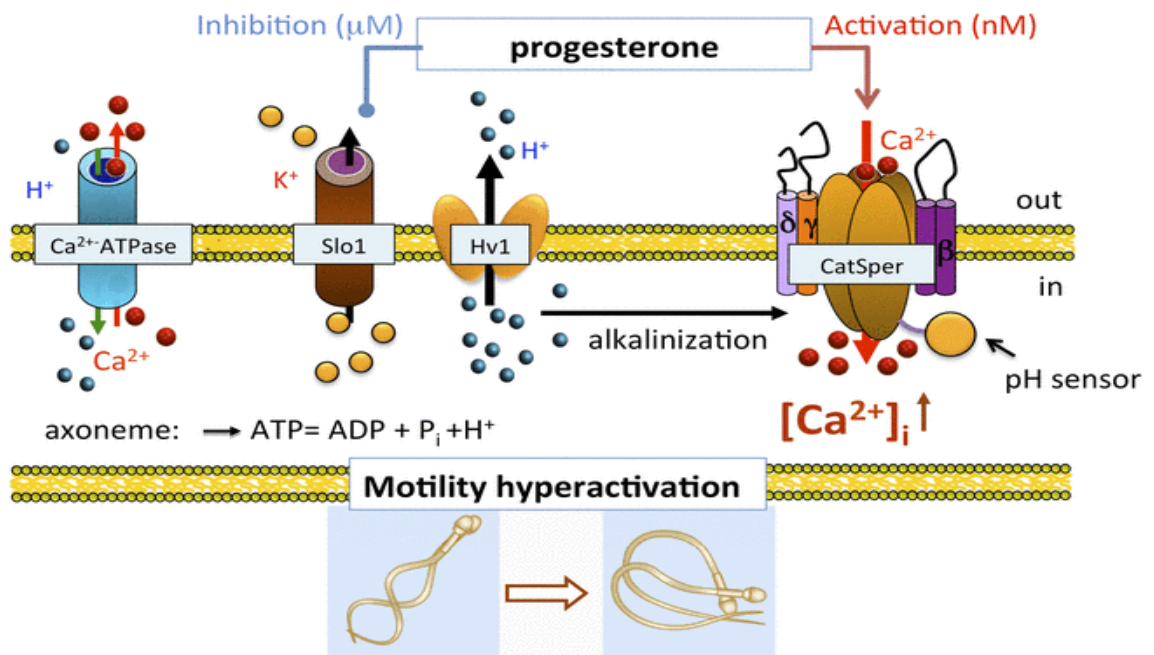
Inactivating mutations of CatSper 1 and 2 have been found in some men (Hildebrand et al., 2010), but the phenotype of these CatSper mutations is unknown. Spermatozoa motility varied between 50% and 0% in these males and their ability to achieve HA or

capacitation was not established (Hildebrand et al., 2010). Tamburrino et al., (2014) indicated that asthenozoospermic males' spermatozoa express on average lower levels of CatSper 1 and that expression of the protein is correlated with the percentage of progressive motility of the semen samples. Expression of CatSper 2 was also found to be reduced in spermatozoa from oligoasthozoospermic men (Bhilawadikar et al., 2013). Tamburrino et al., (2014) concluded that alterations in CatSper genes and protein regulation may occur during spermatogenesis leading to different levels of expression of the functional channel in mature spermatozoa and therefore alterations of cell motility, specifically HA.

### 1.7 Progesterone stimulation of spermatozoa motility

Progesterone is present in the female reproductive tract, is released by cumulus cells surrounding the oocyte and can be found in follicular fluid. Progesterone stimulates a  $\text{Ca}^{2+}$  increase in human spermatozoa, and has been implicated in chemotaxis (Publicover et al., 2007; Teves et al., 2009), and triggering HA (Suarez, 2008).

Progesterone induces this  $\text{Ca}^{2+}$  influx in human spermatozoa by acting through the pH sensitive CatSper calcium channels on the flagellum (Ren et al., 2001; Kirichok et al., 2006) and enhancing the mobilization of stored calcium through calcium induced calcium release (CICR) (Harper et al., 2004) (Figure 1.3). This activation of CatSper by progesterone initiates HA, enhancing spermatozoa functional ability to penetrate and fertilise the oocyte. The very quick physiological response seen in spermatozoa when exposed to progesterone makes it a good agent to use in the *in-vitro* study of spermatozoa functional ability. If spermatozoa show an increased HA movement which increases functional ability to penetrate a viscous media (substitute for fluid found in the female reproductive tract and cells surrounding the oocyte) it is thought that the cells have a higher fertilisation capacity.



**Figure 1.3:** In the female reproductive tract CatSper is partially activated due to intracellular alkalinization and progesterone from cumulus cells of the oocyte. For full activation of CatSper, flagellar plasma membrane must be depolarized. This is achieved by the inhibition of sperm KSper, the channel responsible for membrane hyperpolarization. Spermatozoa encounter P, which inhibits KSper, resulting in membrane depolarization. These events allow full activation of CatSper, trigger sperm hyperactivation, allow spermatozoa to penetrate through the oocyte cell layers, and fertilise (Mannowetz et al., 2013).

### 1.8 Acrosome Reaction

Removal of cholesterol and other sterols from the spermatozoa head surface creates a more fluid membrane which is required for the AR (Osheroff et al., 1999; Bedu-Addo et al., 2005), binding to the ZP (an extracellular coat which surrounds the oocyte and is composed of a matrix of four glycoproteins (ZP1 – ZP4) (Monne et al., 2006), and penetration of an oocyte (Harrison, 1996). Acrosome intact spermatozoa reach the ZP and its components (ZP1 - 4) promote the AR by interacting with spermatozoa surface molecules. Following binding to the ZP3 glycoprotein (Gupta, 2015), outer acrosomal membranes fuse with the spermatozoa plasma membrane. This membrane fusion process exposes the inner acrosomal membrane changing the topology of the plasma membrane and proteins that are essential for sperm-egg recognition and fusion (Inoue et al., 2011). This allows acrosomal contents, such as hydrolytic enzymes (acrosin) to be released in an exocytotic process, which digest the ZP, enabling penetration (Breitbart

& Spungin, 1997; Anifandis et al., 2014). Tyrosine phosphorylation and the activation of multiple kinase pathways, including MAPK and AKT are also involved in the induction of the AR (Sagare-Patil et al., 2012), enabling the membrane altering processes to occur.

The induction of the AR by the binding of spermatozoa to ZP3 induces a series of  $\text{Ca}^{2+}$  dependant events, involving the mobilisation of the acrosome  $\text{Ca}^{2+}$  store in the spermatozoa head and the activation of capacitative  $\text{Ca}^{2+}$  influx from channels on the cell membrane (Blackmore, 1993; O'Toole et al., 2000). Spermatozoa acrosome region contains an inositol 1,4,5-triphosphate-sensitive  $\text{Ca}^{2+}$  channel ( $\text{IP}_3$  receptor), which binds to the second messenger  $\text{IP}_3$  leading to an elevation of  $[\text{Ca}^{2+}]_i$  concentration. It is thought that this is a mobilizable  $\text{Ca}^{2+}$  store. The spermatozoa head also contains a T-type voltage activated calcium channel (Santi et al., 1996) which is released from inactivation by capacitation induced HA of the cell (Arnoult et al., 1998), opening in response to ZP3 induced depolarization (Arnoult et al., 1996) increasing  $[\text{Ca}^{2+}]_i$  and induction of the AR.

The increase in cAMP during capacitation activates actin polymerisation (Buffone et al., 2014). Actin is located in the acrosomal region of the head and in the tail (Fouquet & Kann, 1992) indicating involvement in AR and spermatozoa motility. F-actin breakdown must take place to achieve acrosomal exocytosis (Brener et al., 2003). Megnegi et al., (2015) illustrated that Cofilin, an actin severing protein, controls cellular F-actin, supported by evidence that cofilin is translocated from spermatozoa tail to head during capacitation. Reduced cofilin in the spermatozoa tail maintains a high level of F-actin which is essential for HA motility, whereas the increase in the head enables F-actin breakdown and initiation of AR.



Following the AR, spermatozoa acquire the ability to fertilise the oocyte, exposing previously concealed receptor proteins onto their surface (Okabe, 2013). IZUMO1 is redistributed on the surface of capacitated spermatozoa (Satouh et al., 2012) and Juno is the oocyte receptor expressed on its surface and is essential for female fertility (Bianchi et al., 2014). The interaction between the receptors is necessary for the adhesion of the gametes, their activation and subsequently, fertilisation. After fertilisation the oolemma and the ZP are biochemically altered, ensuring the oocyte is unreceptive to additional spermatozoa therefore reducing the chances of polyspermy (Gardner & Evans, 2006). Juno is rapidly shed from the oocyte cell surface suggesting its involvement in the mechanism of membrane block to prevent polyspermy (Bianchi et al., 2014).

#### 1.8.1 Progesterone stimulating acrosome reaction

Progesterone is also involved in initiating the AR (Suarez, 2008; Tamburrino et al., 2015). Sosa et al., (2015) used progesterone as a trigger, increasing cytosolic calcium relatively quickly (Harper et al., 2003; Darszon et al., 2011) and inducing the swelling of the acrosomal granule that precedes exocytosis; a relatively slow process that takes minutes. When the swelling was complete, fusion pores opened quickly, being triggered by the efflux of calcium from intracellular stores, and so releasing the hybrid vesicles in seconds (Sosa et al., 2015). The ability of follicular fluid to induce the AR is dependent on its progesterone concentration (Morales et al., 1992; Saaranen et al., 1993).

Progesterone is thought to act on voltage operated calcium channels (Blackmore, 1999) or converge with the ZP-activated pathway on activation of store-operated  $\text{Ca}^{2+}$  influx (Barratt and Publicover, 2001).

#### 1.9 Drug Discovery for male subfertility

Drug discovery has been transformed by the introduction of high throughput screening (HTS) of compounds *in-vitro* (Macarron, 2006). The introduction of robotic plate

handling has allowed traditional HTS of a large number of chemicals at a single compound concentration. This method can identify candidates with a desired biological activity (Malo et al., 2006).

Spermatozoa are small complex biological cells, and their physiology is incompletely understood, making it difficult to develop drugs which target specific functions.

However, HTS offers a novel approach to drug discovery. Several screening libraries at the UoD DDU are currently being utilised to develop specific drugs which target spermatozoa and enhance motility and function.

Martins da Silva et al, (2012) developed a novel HTS assay which utilised a FlexStation microplate reader to show that progesterone, a positive control, elicited a reproducible concentration dependent increase in  $[Ca^{2+}]_i$  in human spermatozoa. This new and innovative assay allowed the identification of novel compounds, from the DDU ion channel, chemogenomics and Selleck MIH prescribable libraries, which increase  $[Ca^{2+}]_i$  in human spermatozoa, using progesterone as a positive control and reference of calcium response elicited. Progesterone is known to induce an increase in  $[Ca^{2+}]_i$  which initiates physiological processes required for fertilisation, therefore if drugs act in the same way they would potentially increase cells fertilisation ability *in-vitro*. The use of calcium as a surrogate for motility indicates potential drugs that could induce functional changes in spermatozoa, with potential to be transferred into clinical practice. It is hypothesised that the increase in calcium would induce physiological changes in motility and cell function.

The rational of Drug Discovery is to find a drug or drugs that increase spermatozoa motility characteristics. Asthenozoospermic male spermatozoa samples are main targets for drug treatments. Significantly reduced tyrosine phosphorylation and spermatozoa motility parameters, specifically VAP, VSL, VCL and ALH, can be seen in

asthenozoospermic males when compared with normozoospermic males and males of known fertility (Buffone et al., 2005). Asthenozoospermic males also show a reduced calcium response to progesterone (Espino et al., 2008) and a variation in protein expression in the spermatozoa tail can be seen when compared with normozoospermic spermatozoa (Hashemitabar et al., 2015). Adding drugs to these spermatozoa samples to improve motility characteristics and cell functional abilities, without a negative effect on spermatozoa function – such as an increase in premature AR – would potentially increase ART treatment success, specifically in IUI and IVF cycles.

#### 1.10 Effects of infertility on society and the wider population

The UN Statement on Human Rights states, - ‘Parents have a basic human right to determine freely and responsibly the number and spacing of their children’. Subfertility prevents this fundamental human right. Moreover, increase in rates of subfertility in the context of an increasing aging population creates societal and political anxieties. Increase in dependency ratios has negative effects on the wellbeing of the country where the proportion of the working age population is less than the non-working population (retired, elderly). A reduced working age population, ultimately results in reduced tax income for healthcare, pensions and welfare, and there are robust economic arguments to support investment to fund more ART cycles, because an increase in birth rate will eventually result in a tax gain when these children go into the working population (Hoorens et al., 2007). Infertility also causes great distress to couples affected, strongly impacting on personal relationships, marriages and mental health (Ahmadi Forooshany et al., 2014) therefore an increase in ART success rates could significantly improve the quality of life of affected couples.

The main aims of this project were to determine if 10 compounds from DDU Selleck NIH prescribable library that induced an increase in  $[Ca^{2+}]_i$  in human spermatozoa, seen during the previous Flex Station screen, affect;

1. Spermatozoa motility
2. Spermatozoa functional ability
3. Induction of the acrosome reaction
4. Patient cell response

The initial compound screen was a blinded study using prepared donor sperm (aims 1-3). No information on drug names or mechanisms of action was given until after the experiments were completed. Initial screening was done at single concentration - 30 $\mu$ M - and further screening was done with various concentrations if compounds induced an effect.

Drug research into improving spermatozoa function would be fundamental to countries where IVF and ICSI is not readily available or affordable. Addition of drugs to spermatozoa to improve motility/function and thus chances of conception during an IUI cycle would have a very real impact globally, and a hope for millions of couples.

## **CHAPTER 2**

### **Materials and Methods**

## **Chapter 2: Materials and Methods**

All chemicals used were from Sigma-Aldrich, unless stated otherwise.

### **2.1 Non-capacitating and capacitating media**

Non capacitating buffer (NCB) contained 5mM KCl, 1mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 135mM NaCl, 5mM D-Glucose, 60% sodium DL-lactate ( $\text{CH}_3\text{CH}(\text{OH})\text{COONa}$ ) solution, 20mM HEPES, 2mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  and 1mM Sodium Pyruvate ( $\text{C}_3\text{H}_3\text{NaO}_3$ ), 0.3% BSA. The pH was adjusted to 7.4 by the addition of 1M NaOH with an osmolarity of 290-320mOsm/kg to be physiologically relevant. The same parameters (pH and osmolarity) apply for capacitating media (CM).

CM was adapted from NCB. On each day of experiments, 5ml of 1x NCB was supplemented with 0.3% BSA and 26mM sodium bicarbonate ( $\text{NaHCO}_3$ ). Capacitating media is warmed and gassed in a 5%  $\text{CO}_2$  incubator at 37°C.

*In vivo*, a blood bicarbonate concentration of 24mm (corresponding to approximately 2mg sodium bicarbonate per mL) is in equilibrium with a lung  $\text{CO}_2$  partial pressure of 40mm Hg (corresponding to approximately 5%  $\text{CO}_2$ ) to maintain a physiological pH (7.4). These conditions are implemented *in-vitro* by adding 2mg  $\text{NaHCO}_3$  per mL of medium and maintaining an atmosphere of 5%  $\text{CO}_2$  (Esser, 2010). The  $\text{CO}_2$  level can easily be restored in media with a low buffering capacity, although this type of media is susceptible to rapid acidification by cellular respiration. This can be controlled using bicarbonate in combination with the organic buffer, HEPES (Esser, 2010).

### **2.2 Reagents and Stocks**

Stock solutions of 1% DMSO in distilled water were prepared on a monthly basis and stored at room temperature until use.

Stock solutions for progesterone were prepared by dissolving in ethanol (36mM) and storing in the freezer (-20°C) (source and catalogue numbers see appendix). Final concentration of progesterone used was 3.6 µM (physiologically relevant) (Alasmari, et al., 2013).

Stock solutions for the ten compounds, A-H, were made up by the UoD DDU at a concentration of 10mM solubilised in 100% DMSO using robotics and aliquoted into Eppendorfs. They were tested for purity using LC-MS. Compounds were stored at room temperature (for no longer than 3 months). The final concentration of each of these compounds in spermatozoa suspension was 30µM (same concentration as used in the FlexStation screen), 10µM or 100µM with a final concentration of DMSO not exceeding 1%.

## 2.3 Semen Samples

### 2.3.1 Donor samples

Healthy volunteer donors were recruited following initial screening to determine if their semen was within the WHO parameters for normal fertile males. Semen samples were produced at home by masturbation into sterile plastic containers, after 2-5 days of sexual abstinence. Samples were brought to the laboratory within 1 hour of production and allowed to liquefy at 37°C for 30 minutes. If the sample was already liquefied upon arrival, then it was only incubated for 15 minutes at 37°C.

### 2.3.2 Patient samples

Patient samples were produced at the ACU, Ninewells Hospital prior to their partner's oocyte retrieval, by masturbation into sterile plastic containers after 2-5 days abstinence. In Dundee ACU, commercially available media is used for sperm preparation. Spermatozoa are prepared in the ACU laboratory by DGC using 1.5ml 40%:80%

PureSperm™ (Nidacon, Molndal, Sweden). The 80% pellet is washed by centrifugation at 500g for 10 min in 4ml of SAGE® gamete buffer. Semen samples are assessed by clinical embryologists and allocated to IVF or ICSI according to clinical indications and semen quality. In general, men with approximately  $1 \times 10^6$  motile cells after preparation were allocated to IVF and any men below this were allocated to ICSI. For IVF samples, the supernatant is discarded and the pellet resuspended in Quinn's Advantage® fertilisation medium supplemented with HSA, which supports capacitation. For ICSI samples, the cells are left in 100µl SAGE® gamete buffer. Clinical samples surplus to requirement for IVF or ICSI treatment are used in the lab for research purposes (Alasmari et al., 2013; Tardif et al., 2014).

### 2.3.3 Ethical approval

Written consent was obtained from each individual patient and volunteer sperm donors (males with no known fertility issues) in accordance with the HFEA Code of Practice (version 8) under local ethical approval 13/ES/0091 from Tayside committee of medical research ethics B (see appendix 9.6 for patient and donor consent forms).

### 2.4 Density Gradient Centrifugation

Spermatozoa must be separated from seminal plasma to undergo capacitation, a process essential for fertilisation (Austin, 1951; Chang, 1951). Seminal plasma contains decapacitation factors, bacteria and leukocytes which are harmful to spermatozoa (Mortimer & Mortimer, 2013) therefore cells must be removed from this environment as quickly as possible, ideally within 30 minutes to ensure the least amount of damage (Rodgers, et al., 1983).

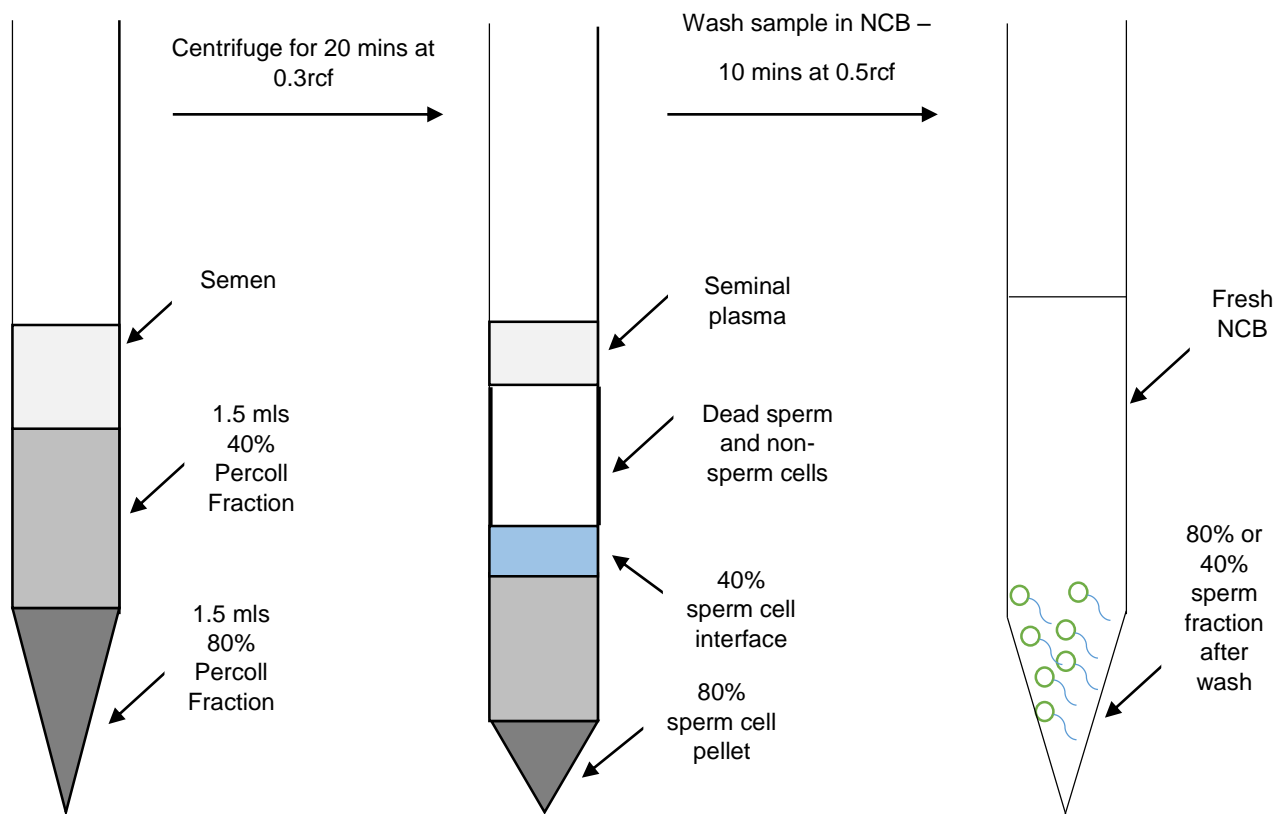
The spermatozoa preparation media, Percoll, is used in the research laboratory. Percoll is a solution used for efficient density separation - it consists of colloidal silica particles which have been coated in polyvinylpyrrolidone (PVP). Due to the specific gravity of



80% (v/v) Percoll colloid -  $\sim 1.10\text{g/mL}$  – only the most mature, morphologically normal human spermatozoa (density  $>1.12\text{g/mL}$ ) can penetrate this bottom gradient layer. The other abnormal spermatozoa collect in the 40% Percoll density gradient layer, which both have lower densities (Mortimer & Mortimer, 2013). Percoll has been replaced in clinics with PureSperm (O'Connell et al., 2003) due to the concerns over its potential toxic effects on spermatozoa (Mousset-Simeon et al., 2004). Percoll gradients are made up by; 90% Percoll (7.2mls Percoll + 0.8mls NCB (10x concentration)), 80% Percoll (4.4mls 90% Percoll solution + 0.6mls NCB (1x concentration)), 40% Percoll (2.2mls 90% Percoll solution + 2.8mls NCB (1x concentration)). All media is warmed to  $37^{\circ}\text{C}$  before use.

1.5ml 80% Percoll was layered under 1.5ml 40% Percoll. The semen sample is layered on top, to a maximum of 2ml semen on each density gradient, and then centrifuged at  $0.3g$  for 20 minutes. After centrifugation the 80% spermatozoa pellet collects at the bottom of the tube. The 40% cell fraction collects above the 80% pellet and below the seminal plasma and dead cells/debris. Debris and supernatant was removed carefully using a pipette. 40% and 80% spermatozoa fractions were removed and washed separately in 4mls NCB by centrifugation at  $0.5g$  for 10 minutes (Mortimer and Mortimer, 2013b) (Figure 2.1). The 40% spermatozoa cell interface was removed and re-suspended in NCB and incubated at  $37^{\circ}\text{C}$ .

The 80% fraction was removed and re-suspended in CM and incubated at  $37^{\circ}\text{C}$  in  $\text{CO}_2$  incubator for at least 2 hours to allow capacitation of spermatozoa.



**Figure 3.1: Schematic diagram of the stages of density gradient centrifugation.** Tube 1 indicates how the media is layered, before the tube is placed in the centrifuge and spun for 20 minutes at 0.3rcf. Tube 2 indicates how the media is layered after centrifugation. The seminal plasma and dead sperm and non-sperm cells are removed and disposed of. The 40% and 80% cell fractions are removed and separated into different tubes with fresh NCB where they are centrifuged for 10 minutes at 0.5rcf.

## 2.5 Evaluating Spermatozoa Characteristics

All motility analysis was done using Computer-assisted sperm analysis (CASA)

[CEROS machine (version 12), Hamilton Thorne Research, Beverly, MA, USA]

attached to an external microscope, to determine semen characteristics and prepared

spermatozoa parameters. CASA images spermatozoa by identifying the head and tail

(Mortimer, 2000). The kinematic values determined for each spermatozoa cover the

velocity of movement, the width of the spermatozoa heads trajectory and the frequency

of the change in direction of the head. Velocity values include; VCL - total distance that

the spermatozoa head covers in the observation period, VSL - determined from the

straight-line distance between the first and last points of the trajectory and gives the net

space gain in the observation period, and VAP - the distance the spermatozoa has travelled in the average direction of movement in the observation period.  $LIN = (VSL/VCL) \times 100$ ; a measure of the departure of the cell track from a straight line.  $STR = (VSL/VAP) \times 100$ ; a measurement of the departure of the cell from a straight line. The ALH is the width of the lateral movement of the spermatozoa head. It is calculated as the total width of the head trajectory

Spermatozoa kept at 37°C, were measured on MicroCell 20µm deep counting slides. 4µl of sample was covered with a cover slip, and left on the heated stage until cells stopped drifting. For initial semen concentration analysis and prepared sample count approximately 1000 cells from 3-4 different fields of view were counted to obtain a sample concentration. If the cell concentration was too high for CASA to analyse (>100M/ml), the sample was diluted (1 in 10) using NCB (90 µl NCB, 10 µl semen sample). When the concentration is too high and there are too many spermatozoa in the field, it is not possible for CASA to reconstruct their trajectories accurately (Mortimer, 2000).

Playback feature was used after each recording to confirm that >95% spermatozoa heads had been detected. The CASA machine follows how the head is moving rather than the flagellum because the frequency of the flagellar beats is very high and move too quickly for standard video system analysis. CASA detects head shape and size therefore discounts any debris and other cells present. A negative phase contrast objective was used to assess spermatozoa motion characteristics, as previously described (Alasmari et al., 2013). System parameter settings were: 30 frames at 60 frames per second (Hz); minimum contrast 80; minimum size 3 (pix); upper and lower gates of 3.39 and 1.4 for intensity; and 0.85µm and 4.24µm for size. The default values for non-motile cells were 6µm and 160 for size and intensity, respectively. A minimum of 16 data points were used for tracking a cell. Spermatozoa with a VAP >25µm/s and

80% STR were considered progressive, and these spermatozoa are thought to be better able to penetrate cervical mucus *in-vitro* (Aitken RJ et al, 1985; Mortimer D et al, 1986; Mortimer D. 1994; Bjorndahl L et al, 2010). For human spermatozoa analysis at 60 Hz, the definition for HA motility is a VCL > 150µm/s, ALH > 7.0µm and decrease in LIN <50% (Mortimer et al, 1998).

During motility experiments (Chapter 2.7), 3µl spermatozoa were added to single use 20µM deep four chamber CASA slides (Vitrolife, Sweden). At each time point, aliquots were taken from control and treatment groups and loaded on to the 4 chamber slides. The kinematics of a minimum of 200 cells were counted per frame, with a minimum of 4 frames counted per chamber (Tardif et al., 2014), giving a minimum total of 800 motile spermatozoa counted (unless otherwise stated) in every experiment, to get an accurate reading.

2.6 Flex Station Assay Screen (experiments conducted by Sarah Martins da Silva in collaboration with the Dundee Drug Discovery Unit (Martins da Silva et al, 2012))

Spermatozoa samples (3-4 donors) were collected and prepared (by DGC) as previously described (Chapter 2.4). Spermatozoa were then subjected to capacitating conditions for 2 hrs 30 minutes. Media and supernatant were removed and cells re-suspended in Flex Station assay buffer (1xHBSS, 20mM HEPES, 0.5mM probenecid, pH 7.4) and concentration and volume were determined using CASA. Approximately 110 million cells constituted in a 5ml volume were added to an equal volume of Ca<sup>2+</sup> sensitive dye, Calcium 3, which had been made up to twice the manufacturer's recommended concentration. Cells were incubated at 37°C for 60 minutes, then recovered by centrifugation at 700g for 5 min at room temperature and re-suspended in Flex Station assay buffer at a concentration of 5 x 10<sup>6</sup> cells/ml. The cells were plated out onto 384 well clear bottom, black wall plates at a concentration of 2.5 x 10<sup>5</sup> cells/50µl/well. The plates were then centrifuged at 700g for 5 minutes at room temperature to ensure the

cells were at the base of each well. A plate was made up to contain test compounds (from 3 DDU libraries) at a 5x final concentration, with the use of 10 $\mu$ M progesterone as a control (all compounds plated by robotics at DDU). Both the assay and the plates containing compounds along with FLIPR tips were placed into the appropriate compartment of the Flex Station. The Flex Station recording was started and 12.5 $\mu$ l of test compound was added after 18 seconds(s). Ca<sup>2+</sup> dependent fluorescence of the Calcium 3 dye was measured for 100s as recommended by the manufacturer (excitation wavelength = 485nm, emission wavelength = 525nm, cut-off = 515nm). Hit compounds – compounds which indicated a significant increase in [Ca<sup>2+</sup>]<sub>i</sub> - were identified by software analysis of the area under the curve function when compared to progesterone (positive control). This project examined 10 of these compounds for any effect the induction of [Ca<sup>2+</sup>]<sub>i</sub> may have on motility and functional ability of spermatozoa (see below).

## 2.7 Motility screen

A single semen sample was used on each experimental day. Following preparation the 40% and 80% fractions were divided to test for motility effects of compounds and controls – 40% cell fraction in NCM and 80% cell fraction in CM. The number of compounds tested each day depended on the concentration of cells obtained from DGC. 97 $\mu$ l sperm sample – at a concentration of 20M/ml - were aliquoted out into Eppendorfs and the compounds were added. Compounds were initially screened at 30 $\mu$ M – the same as in the initial high-throughput Flex Station screen. 1% DMSO (final concentration) was used as a vehicle control. Spermatozoa with no compound or DMSO were used as a negative control. Spermatozoa were analysed at 0 minutes (T0), 15 minutes (T15) and 30 minutes (T30) using CASA microscope. Cells were analysed at T0 to determine immediate effects of the compound at the initial time of exposure. By T30, the optimal effects of the compounds that initiated a change in motility were observed and had

reduced so the effects of a longer incubation period were not recorded. 3µl of spermatozoa suspension was then loaded onto preheated fixed chamber Microcell slides and CASA analysis conducted (Chapter 2.5). All compounds were screened at a final concentration of 30µM for effects on 40% (non-capacitated conditions) and 80% (subjected to capacitating conditions) cell fractions. Of the compounds that showed an increase in motility parameters, these were then also tested at 10µM and 100µM final concentration, on both 40% (non-capacitated) and 80% (capacitated) spermatozoa fractions to determine if this caused greater motility effects on the cells.

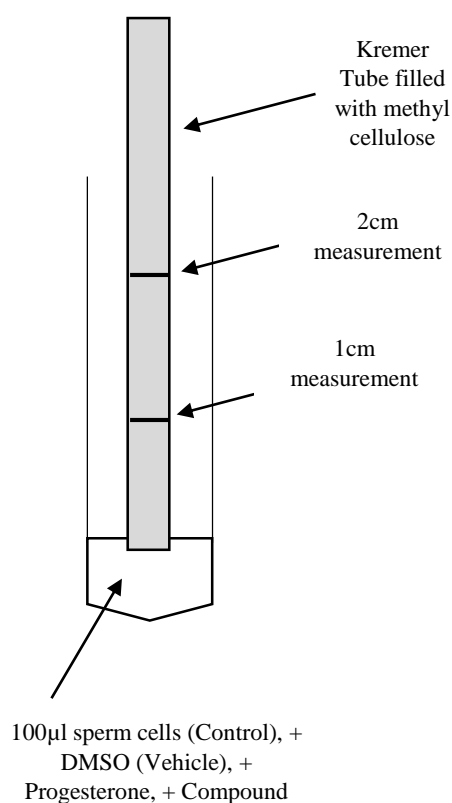
## 2.8 Kremer penetration test

10% MC (an artificial cervical mucus substitute) was prepared using 20mls CM and 0.2g MC. Following brisk shaking for 1 minute, the mixture was left on the automated mixer for 1 hour, then kept in the fridge overnight, removing all of the bubbles. Before use, the MC mixture was incubated in the CO<sub>2</sub> incubator for 3 hours to allow it to equilibrate. MC has a viscosity of 4000cp (MC4000).

As previously described (Ivic, et al., 2002), Kremer tubes (0.4mm depth, 4mm width) (Camlab Limited Cambridge UK) were filled with 10% MC by placing them in Eppendorfs containing 1ml of MC mix and placing for 30 minutes in a CO<sub>2</sub> incubator at 37°C until they were filled. External MC was then wiped off and the tube tops sealed with plasticine to prevent evaporation. The filled tubes were subsequently placed into Eppendorf tubes containing i) 100µl of spermatozoa (control), ii) 100µl spermatozoa and 1% DMSO (vehicle control), iii) 100µl spermatozoa and 3.6µM progesterone (positive control) or iv) 100µl spermatozoa and 30µM compound A–J (Figure 2.2). The cell concentration used was standardized to  $10 \times 10^6$  ml, thus 1 million cells in each Eppendorf tube. This cell number is sufficient to give a good indication of viscous media penetration abilities (Chapter 4). Too many cells result in spermatozoa colliding

and moving in the wrong direction due to overcrowding, whilst too few cells will not give an accurate representation of functional motility. The Eppendorfs containing Kremer tubes were incubated at 37°C in 5% CO<sub>2</sub> for 1 hour. The Kremer tubes were then removed, the bottom sealed with plasticine to prevent the cells from leaking out and any residual spermatozoa wiped off of the surface. Cells at 1cm and 2cm were then counted using an Olympus CX41 microscope (20X objective final magnification x200) (Olympus Corporation, Tokyo, Japan), by focusing on sequential depths until all planes (and cells) in that section had been counted. This was done 4 times over the length of the tube at 1cm and 2cm from the base. Of the 4 counts, an average was taken to give a cell number at 1cm and 2cm for each Kremer tube.

These values were subjected to statistical analysis (Chapter 2.10) – specifically ANOVA tests - to compare control, vehicle and drug treatment and to determine if there was an effect on cell penetration into viscous media after exposure to compounds. Results were normalised to parallel untreated controls to allow comparison between different experiments.



**Figure 2.2: Schematic diagram of Kremer test set up used in this study.** Kremer tube (0.4mm depth, 4mm width) filled with MC and marked at 1cm and 2cm from the bottom, was placed in an Eppendorf filled with 100µl sperm cells (Control), 100µl sperm cells and DMSO (Vehicle), 100µl sperm cells and progesterone or 100µl sperm cells and compound.

## 2.9 Acrosome Reaction Assay and Flow Cytometry

An assay was developed to detect the occurrence of the AR in human spermatozoa - after exposure to hit compounds that induced an increase in  $[Ca^{2+}]_i$  – using Flow cytometry (FCM) analysis. FCM is an experimental technique used to measure physical and multicolour fluorescent properties of cells flowing in a moving stream. A cell suspension flows through a tubular fluidics system and is exposed to a laser which illuminates different areas of the particle. The recorded emissions from the laser illumination of the cells is digitalised and computer handled, using Flow Jo software, to provide visual information about a cell sample.

Spermatozoa acrosome was studied by staining with lectin from *Pisum sativum* (pea) labelled with Fluorescein isothiocyanate (PSA-FITC) as described by Zoppino et al., (2012). Calcium ionophore A23187, a known inducer of the AR, was used as a positive control.

Donor spermatozoa were prepared by DGC as described previously (Chapter 2.4). The FCM assay was performed in standard Flex Station assay buffer. Cells were diluted to  $2 \times 10^6$ /ml in HBSS +5mg/ml BSA. 1.5ml aliquots of cells were added to FCM tubes. TO-PRO3, a fluorescent nuclear and chromosome counterstain that penetrates dead cell membranes was added to each FCM tube (5mg/ml stock – final concentration 5µg/ml; 1.5µl added per tube). PSA-FITC binds to acrosomal content and was used to determine cells which have undergone AR (1mg/ml stock – final concentration 5µg/ml: 7.5µl added per tube). FITC has excitation and emission peak wavelengths of approximately 495 nm/519 nm. TO-PRO3 has excitation and emission peak wavelengths of approximately 642 nm/661 nm. Background TO-PRO3 and FITC fluorescence was initially assessed by FCM, and compounds were then added to the FCM tubes. All ten compounds (A-J) were assessed at 30µM, 1% DMSO as a vehicle control, 10µM



A23187 (calcium ionophore) as positive control, and ethanol as vehicle control. FCM recordings were taken at time 0 minutes (T0). Cells were incubated at 37°C for 1 hour. FCM recordings were taken again at time 60 minutes (T60). 10,000 events were analysed at each recording. Data was analysed using the Flo Jo software

## 2.10 Statistical Analysis

GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA) was used for all statistical analysis in this research. Statistical significance was considered  $P \leq 0.05$ . Normality of data was assessed using frequency distributions and Shapiro-Wilk normality test. All results were expressed as mean  $\pm$  SEM (standard error of the mean).

Analysis of variance (ANOVA) and T-test were used for statistical comparison of normalised/parametric data, or data normalised after transformation. Tukey test was used post ANOVA to indicate statistical comparisons between groups. Kruskal-Wallis one way ANOVA and Mann-Whitney U test were used for statistical comparison of data that was unable to be transformed into normally distributed data. Dunns test was used post Kruskal-Wallis to indicate statistical comparison between groups. Pearson Correlation Coefficient was used to measure the strength and direction of the linear relationship between x and y data sets.

## **CHAPTER 3**

# **CASA Motility Screen on 10 Hit Compounds**

### **Chapter 3: CASA Motility Screen on 10 Hit Compounds**

#### **3.1 Introduction**

A British Fertility Society review concluded that the chance of natural conception increases with increasing numbers of motile spermatozoa in the ejaculate (Tomlinson et al., 2013). Cumulative conception rates correlate with increased percentage motility in the spermatozoa sample, specifically the significance increases with high velocity (Grade a) motile spermatozoa (velocity  $>25\mu\text{m}/\text{sec}$ ) (Larsen et al., 2000). In humans' *in-vivo*, spermatozoa are deposited into the anterior vagina at coitus. They rapidly make contact with cervical mucus and enter the cervix where spermatozoa selection begins (Rath et al. 2008). Spermatozoa move through the uterotubular junctions and fallopian tubes, toward the oviduct where one cell fertilises the oocyte. One of several selection processes is motility; the spermatozoa that can swim faster and most efficiently past all of the physiological barriers to reach the oviduct can fertilise the oocyte (Suarez and Pacey, 2006). Motility is also a major influence in ART and enhancing spermatozoa motility is a priority to improve outcome of the techniques used, specifically in IUI and IVF. Theoretically if compounds induced an increase in spermatozoa motility parameters such as HA and progressive motility in IVF and ICSI respectively, this could increase fertilisation rates and the chance of a live birth for infertile couples.

##### **3.1.1 Spermatozoa motility and ART success rates**

IVF relies on certain conditions for its success; a substantial number of functional spermatozoa and adequate sperm-oocyte interaction, which is hindered in male patients with dysfunctional spermatozoa (Tournaye et al., 2002). As a result, men with severe spermatozoa dysfunction, are recommended to undergo ICSI because characteristics such as motility and concentration are not major factors in the fertilisation success of this technique. Now, many clinics use ICSI as a first line treatment for all couples with

male factor infertility because of its higher success rates (HFEA, 2013), but the downsides are that it is more expensive (Wu, et al., 2014), invasive and removes all chance of physiological sperm selection by the oocyte (Charehjooy et al., 2014). As ICSI bypasses the natural selection process occurring during fertilisation, this raised concerns that spermatozoa with fragmented or damaged DNA may be picked to fertilise an oocyte *in-vitro* (Hourcade et al., 2010). The integrity of spermatozoa DNA is vital for paternal reproductive potential, and it has been indicated that cells from infertile males have a higher incidence of DNA damage (Sergeie et al., 2005; Zini et al., 2005). In mice, a number of genetic and epigenetic alterations, such as; delayed male pronucleus demethylation, telomeres of various sizes, alterations of gene expression in blastocyst stage embryos and modification of the expression of imprinting genes, were evident in preimplantation embryos after ICSI using DNA fragmented spermatozoa (Fernandez-Gonzalez et al., 2008). Also as ICSI was introduced in 1992 (Palermo et al., 1992), most children conceived by this technique are not yet old enough to determine their reproductive potential.

A study by Tournaye et al., (2002) hypothesised that by increasing spermatozoa concentration of motile cells in IVF cycles, this would improve fertilisation rates and reduce the need and use of ICSI. The study compared success rates of IVF cycles - using a high (800,000 motile sperm/ml) or low (200,000 motile sperm/ml) insemination concentration - with ICSI cycles. The male partners' spermatozoa was used and the female oocytes were split for IVF and ICSI comparison. When comparing low concentration IVF with ICSI, overall fertilisation rate was significantly lower after IVF (37.4% vs 64.3%). In the high spermatozoa concentration IVF vs ICSI comparison there was no significant difference in overall fertilisation rate (59.6% vs 67.6% respectively) (Tournaye et al., 2002). Although high spermatozoa concentration IVF has been

reported to be successful it is not used in clinical practice, possibly because of potential increased risk of polyspermy.

Donnelly et al., (1998) found that when comparing semen samples that were used in IVF procedures, when  $\geq 50\%$  oocyte fertilisation and pregnancy rates were observed, the spermatozoa samples had a significantly greater percentage progressive motility in both the semen and prepared samples compared with spermatozoa from cycles which achieved  $< 50\%$  oocyte fertilisation and pregnancy rates. VAP, VSL and VCL were also significantly increased in the spermatozoa samples which produced  $\geq 50\%$  oocyte fertilisation. ALH was significantly lower in prepared spermatozoa from cycles which achieved  $< 50\%$  fertilisation after IVF cycles. Also, the percentage of HA spermatozoa was significantly lower in failed fertilisation IVF cycles (Donnelly et al., 1998). This reduced percentage of fertilisation rates could be because the spermatozoa are unable to penetrate the ZP and oocyte due to reduced HA and ALH, therefore by targeting these motility parameters using compounds *in-vitro*, there is potential to increase fertilisation rates during IVF cycles.

British Fertility Society review concluded that motile spermatozoa influences the outcome of IUI, suggesting a concentration of  $5 \times 10^6$  motile spermatozoa is the minimum requirement for reasonable success (Tomlinson et al., 2013). It has been shown that when low numbers of motile spermatozoa ( $1 \times 10^6/\text{ml}$ ) are used for insemination in IUI cycles, success rates are very low (Campana et al., 1996; Berg et al., 1997). If spermatozoa were incubated with compounds that increase motility parameters, when they were inseminated into the female reproductive tract there would theoretically be an increased chance of cells reaching the fallopian tube and fertilising the oocyte, therefore increasing success rates of IUI.

### 3.1.2 Previous studies using compounds *in-vitro*

The concept of adding drugs to improve spermatozoa motility is not novel, and has been researched for many years. There are various physiological mechanisms in spermatozoa, many not well understood, that could be potential targets for motility enhancing compounds, and ways of improving this motility *in-vivo* and *in-vitro* have been subject to extensive research (Wald, 2008). Before the widespread use of ICSI in ART, non-specific PDE inhibitors, such as pentoxifylline (PTX), were used in fertility clinics to improve IVF rates through chemical stimulation of spermatozoa. PTX improved motility parameters *in-vitro* in normospermic and asthenozoospermic samples (Yunes et al., 2005). A significant enhancement of fertilisation rates was shown after PTX use and 77 pregnancies were achieved (Yovich, 1993). PTX significantly increased the speed of spermatozoa but did not affect the total percentage motile cells in the sample. However, a significant disadvantage from using PTX was its premature induction of the AR (Yovich, 1993), which discouraged its use in clinical settings. Also previous studies using PTX indicated it had a detrimental effect on mouse embryo development (Tournaye et al., 1993).

Sildenafil citrate (Viagra), another PDE inhibitor, is a prescribable drug that treats erectile dysfunction, and acts via the inhibition of type 5 PDE. It has been reported to increase spermatozoa motility, capacitation, and protein tyrosine phosphorylation but does not induce the AR of human spermatozoa (Lefievre et al., 2000).

Melatonin receptors have been found on spermatozoa and so it was proposed that this may be involved in modulating spermatozoa activity (Van Vuuren et al. 1992).

Evidence suggests that melatonin, as well as its metabolites, are involved in the protection of various cell types against damage induced death, likely due to their antioxidant effects (Tan et al 1993; Hardeland et al 2009; Espino et al. 2010). It is

hypothesised that melatonin may protect spermatozoa from oxidative stress induced apoptosis (Espino et al., 2010).

The possibility to develop therapeutic compounds to enhance spermatozoa motility is thus not a novel idea. Evidence supports the idea of using compounds to induce motility as it has been previously indicated that it proves successful, although work must be done to ensure the safety and non-toxicity of these compounds. Analysis of other effects of novel pro-motility compounds *in-vitro* is clearly important and another aim of this project.

### 3.1.3 Replacement of ICSI by increasing IVF/IUI fertilisation rates

Implementation of conventional IVF to replace the invasive ICSI procedure, as recommended by WHO, would be economically beneficial (WHO, 2000). A study using Fluorescent in-situ hybridisation (FISH) to determine chromosomal abnormalities at the first cleavage division by analysing 3PN zygotes, indicated a statistically significant increase in chromosomal abnormalities occurring in ICSI zygotes (7.4%) compared with IVF (1.5%) (Macas et al., 2001). This evidence suggests that the absence of natural spermatozoa selection in the ICSI procedure produces potential detrimental effects on the embryo. The IVF procedure still allows for the physiological selection of spermatozoa that occurs *in-vitro*, potentially reducing chromosomal abnormalities and supporting its use in ART. The available evidence collected over the years suggests that the liberal use of ICSI in couples without a clear severe male factor is not supported (Tomlinson et al., 2013).

In summary, by improving spermatozoa quality directly, it is hypothesised that the implementation of ART can either be avoided or down-graded. For example IVF can be substituted for IUI (Goverde et al., 2000), or conventional ICSI can be substituted for IVF. In many couples faced with infertility, specifically male factor oligozoospermia

and asthenozoospermia, there is currently no choice between IVF and ICSI. (Tournaye et al., 2002). It is hoped that new drug regimens, as explored in this project, can be developed and tested to enhance spermatozoa function (Lefievre et al., 2007).

Development of novel therapeutics to enhance spermatozoa motility and function, and potentially fertilisation and live birth rates, would also make fertility treatments more widely available to the global population; for example replacing IVF with IUI would make treatments more affordable for infertile couples worldwide where the cost and infrastructure required for ART is a limiting factor.

Finding novel therapeutics for male factor subfertility that can be used *in-vitro* to improve success rates of ART is a very valuable research area. The main aim, from a clinical perspective, is to increase the number of functional spermatozoa surrounding the oocyte *in-vitro*, as it is believed this would increase the chance of fertilisation (Publicover & Barratt, 2011), and increasing motility is a key factor in achieving this. This project screened 10 compounds from the UoD DDU known to induce an increase in  $[Ca^{2+}]_i$  - which *in-vivo* correlates with increases in motility and other physiological processes which increase a cell's fertilisation potential - to determine if this process stimulates donor spermatozoa motility parameters as a result of the increase in  $[Ca^{2+}]_i$ .



### 3.2 Experimental Design

Semen was prepared by DGC (Chapter 2.4). The pellet from the 80% and 40% fractions were separated; 80% into CM and incubated for 2-3 hours in CO<sub>2</sub> incubator at 37°C to allow cells to capacitate, and 40% into NCB and incubated for 30 minutes at 37°C.

Non-capacitated spermatozoa were substitutes for ICSI cells and capacitated spermatozoa were substitutes for IVF, as this is the media in which the cells are prepared in clinical practice (Chapter 2.3.2). Also, ICSI spermatozoa are very poor quality and the 40% fraction is a reasonable substitute for these cells. Baseline sperm motility parameters were measured and recorded using CASA (Chapter 2.5) before exposure to compounds A-J. 97µl spermatozoa was aliquoted out into Eppendorfs and 3µl compounds (1mM concentration after 1 in 10 dilution; original stock concentration 10mM in 100% DMSO) was added. Compounds were initially screened at 30µM final concentration – the same as the initial high-throughput Flex Station screen. 1% DMSO was used as a vehicle control. Spermatozoa were analysed at 0 minutes (T0) (the time of drug or control addition), 15 minutes (T15) and 30 minutes (T30) using CASA microscope and computer software by counting a minimum of 200 cells four times for each slide (minimum of 800 cells were analysed each time) as previously described (Chapter 2.5). Specific motility parameters were analysed; % HA (80% capacitated cells only), % progressive, % motile, % rapid, VAP, VCL, ALH, % LIN and % STR. Compounds that showed positive effects on any motility parameters were subsequently tested at 10µM and 100µM.

Normality of the data was determined using the Shapiro-Wilk normality test. Data was transformed if necessary. ANOVA (analysis of variance) test was used for statistical comparison of normalised/parametric data, or data normalised after transformation. Tukey test was used post ANOVA to indicate statistical comparisons between groups.

### 3.3 Results

In summary of the results from the 30 $\mu$ M 80% and 40% spermatozoa fraction screen, drugs A and B showed no statistically significant difference between any drug treated and control samples when analysing 9 different motility parameters in both fractions (Figures 3.1; 3.2; 3.11; 3.12). Drugs C, F and H showed no statistically significant differences at 30 $\mu$ M on the 80% spermatozoa fraction, but on analysis of the % HA graphs (Figures 3.3; 3.6; 3.8) there was an increase in this spermatozoa motility parameter after exposure to each compound when compared with control samples. Drugs C and H also showed no significant differences on motility parameters in the 40% cell fraction (Figures 3.13; 3.18) although drug H results indicated an increase in % progressive motility, but this was not statistically significant. Drug F 30 $\mu$ M appears to be having negative effects on the 40% cell fraction (Figure 3.16), as at T0 ( $P=0.0045$ ) + T30 ( $P<0.05$ ), the % progressive cells were significantly less after exposure to the compound when compared with controls. With regards to % motility ( $P=0.0434$ ), % rapid ( $P=0.0209$ ), and % straightness ( $P=0.0132$ ), at T0 there was a significant decrease in drug F treated cells compared with control. The decrease suggests that the compound is having an immediate effect on non-capacitated cells but this weakens over time and motility goes back to control levels. For all motility parameter results see appendix 9.2.

Drugs D, E and G appeared to be having detrimental effects on motility parameters in both 80% and 40% cell fractions. The 80% spermatozoa cell fraction exposed to drug D for 30 minutes showed a statistically significant reduction in % HA at all three time points (T0;  $P<0.05$ , T15;  $P=0.0017$ , T30;  $P=0.0017$ ) when compared to control and DMSO treated cells (Figure 3.4). When analysing % progressive cells, only at T30 was there a significant decrease in drug treated cells ( $P=0.0211$ ). Motility, a good indicator

of cell viability, indicated at all three time points a significant reduction in drug D treated samples (T0;  $P < 0.01$ , T15;  $P < 0.05$ , T30;  $P < 0.0001$ ). A statistically significant decrease in % rapid cells (T0;  $P = 0.0045$ , T15;  $P < 0.01$ , T30;  $P < 0.0001$ ), VAP (T0;  $P = 0.0028$ , T15;  $P = 0.0002$ , T30;  $P < 0.01$ ) and VCL (T0;  $P = 0.0023$ , T15;  $P < 0.0001$ , T30;  $P = 0.0003$ ) was seen at all time points in cells treated with drug D. At T15 only, there was a significant decrease in ALH between drug D treated and control cells ( $P = 0.0362$ ). After exposure of 40% spermatozoa fraction to compound D for 30 minutes, 2 motility parameters out of 8 were significantly affected. % progressive cells at T0 were statistically significantly reduced in drug treated cells ( $P = 0.0166$ ), and VAP at T30 was statistically less in drug D treated cells ( $P = 0.027$ ) than controls (Figure 3.14). See appendix 9.2 for other motility parameter results. These results would suggest that drug D is having a detrimental effect on spermatozoa motility in both 80% capacitated and 40% non-capacitated spermatozoa fractions, so no further analysis was undertaken.

In 5 of the 9 motility parameters analysed, there is no statistically significant difference between drug E treated 80% cells and control samples (see appendix 9.2 for other motility results). At T30 there was a statistically significant decrease in the % rapid cells ( $P = 0.0153$ ) seen in the drug E treated cells (Figure 3.5). On analysis of VAP ( $P < 0.05$ ) and VCL ( $P = 0.042$ ), at T15 there was a statistically significant decrease in drug E treated samples. There was a significant decrease in ALH at T0 ( $P = 0.0173$ ) in the drug E treated sample. On analysis of 40% non-capacitated spermatozoa fraction after exposure to drug E at  $30\mu\text{M}$  for 30 minutes (Figure 3.15), only 1 motility parameter was affected. VAP at T15 ( $P = 0.0395$ ) indicated a statistically significant decrease in drug treated cells compared with control. For other motility parameter results see appendix 9.2. Drug E appears to have negative effects on spermatozoa motility, suggested by results from the exposure to both 40% and 80% cell fractions.

On analysis of 80% spermatozoa fraction motility parameters after exposure to drug G (Figure 3.7), the results illustrated that at T15 ( $P=0.0422$ ) and T30 ( $P=0.0324$ ), there was a significant decrease in HA in drug treated cells when compared with control. When analysing % rapid cells, the only significant difference was seen at T30 ( $P=0.0337$ ), with a decrease after drug treatment. VAP (T0;  $P=0.0378$ , T15;  $P=0.0130$ , T30;  $P=0.0186$ ) and VCL (T0;  $P=0.0150$ , T15;  $P=0.0094$ , T30;  $P=0.0117$ ) were both statistically significantly decreased at all time points and only at T0 was there a significant decrease in ALH ( $P=0.0324$ ). See appendix 9.2 for other motility parameter results. On analysis of drug G 30 $\mu$ M treated 40% cell fraction when compared with controls (Figure 3.17), only two motility parameters illustrated a significant difference. At T0 ( $P=0.013$ ) and T15 ( $P=0.0115$ ), there was a significant reduction in VAP after drug treatment, but no difference at T30. Only at T15 ( $P=0.0161$ ) was a significant reduction in VCL. Although not statistically significant, the trend indicates a decrease in % progression, % rapid and % motility. See appendix 9.2 for other motility parameter results. The results would suggest a detrimental effect of compound G on both 80% and 40% spermatozoa viability.

In the 80% spermatozoa fraction, of the 9 motility parameters analysed, drug I only had significant effects on 3 (Figure 3.9). At T0, there was a significant decrease in % progressive cells after treatment with drug I when compared with control ( $p=0.0357$ ). When analysing both % motile (T0;  $P=0.0262$ , T15;  $P<0.05$ , T30;  $P=0.0012$ ) and % rapid cells (T0;  $P=0.0207$ , T15;  $P=0.017$ , T30;  $P=0.0053$ ), it was illustrated that at all three time points there was a statistical decrease in drug I treated cells. On analysis of 40% spermatozoa fraction exposed to 30 $\mu$ M drug I, three motility parameters were significantly altered when comparing drug treated cells to controls (Figure 3.19). % progressive (T0;  $P=0.0355$ , T30;  $P<0.05$ ) and % straightness (T0;  $P=0.0318$ , T30;

$P=0.0194$ ) of cells indicated a significant decrease in drug I treated at T0 and T30, but no difference at T15. On analysis of % linearity it was seen that at T15 ( $P=0.0335$ ) there was a statistically significant decrease between drug treated cells and controls. This indicates a detrimental effect of drug I on 80% and 40% non-capacitated cells. See appendix 9.2 for other motility results. There was no further analysis done on these drugs.

Finally drug J showed no significant difference in any motility parameters analysed after exposure to 80% and 40% spermatozoa fractions (Figure 3.10; 3.20), therefore suggesting it is having no effect on motility or cell viability (see appendix 9.2 for other motility results).

On analysis of the 10 $\mu$ M and 100 $\mu$ M screen of selected compounds on 40% cell fraction it was observed that both C and F at both concentrations showed no significant increase or decrease in any motility parameter studied (Appendix 9.2). Drug H showed no significant difference at 100 $\mu$ M (Appendix 9.2) but at 10 $\mu$ M concentration there was a significant increase in % linearity at T15 ( $P<0.05$ ) (Figure 3.21). Although this is a positive effect, it wears off by T30 so there is not enough evidence for further analysis of this compound (see appendix 9.2 for other motility results).

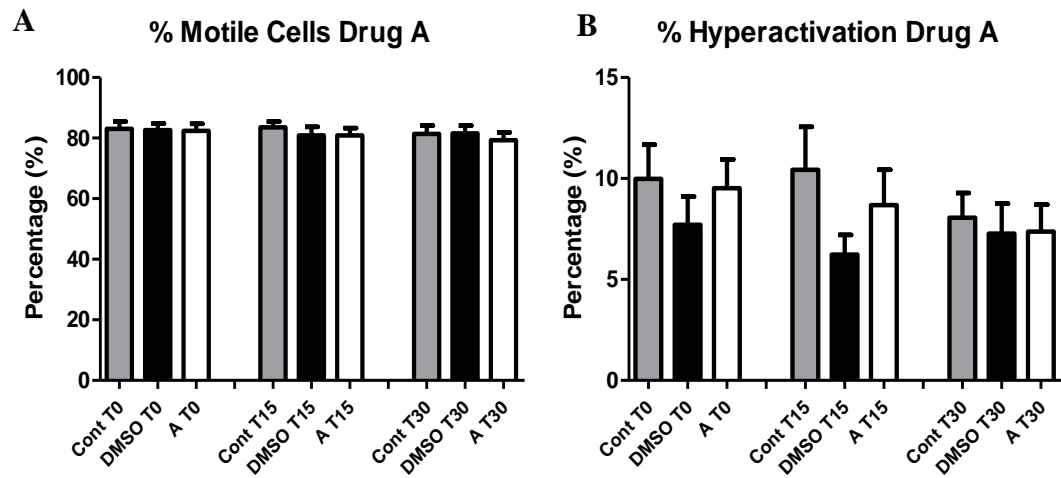
After screening the 80% cell fractions with compounds C, F, and H at 10 $\mu$ M (Appendix 9.2), no significant difference in any cell motility parameter was observed. However, when screening the same compounds under the same conditions at a concentration of 100 $\mu$ M, there were some significant affects. Drug C showed the most positive effects, with a significant increase in HA seen at all time points (T0;  $P=0.0003$ , T15;  $P<0.0001$ , T30;  $P<0.01$ ) and significant increase in VCL at T0 ( $P<0.05$ ) and T15 ( $P<0.01$ ) (Figure

3.22). There was a significant decrease in % progressive cells ( $P=0.0173$ ) and increase in ALH ( $P=0.0116$ ) at T0, which correlates with the significant increase in HA cells as they would move in a less progressive fashion and their head movements would be very vigorous. Drug F also indicated an increase in HA cells but this was only significant at T15 ( $P<0.01$ ) although the trend indicated an increase at all time points (Figure 3.23). There was a significant increase in ALH at T15 ( $P<0.05$ ) which correlates with the increase in % HA. By T30 there was a significant decrease in % motility ( $P<0.01$ ) and % rapid ( $P<0.05$ ) cells. There was no positive effects of drug H after incubation at  $100\mu\text{M}$  (Figure 3.24). There was a significant decrease in % motile ( $P=0.0403$ ) and % rapid cells ( $P=0.051$ ) at T30. This decrease could indicate that over time drug H is having detrimental effects on sperm cells motility at higher concentrations.

In no experiment is there a statistically significant difference between control and DMSO suggesting that DMSO – the vehicle control - is not having any effects on spermatozoa motility.

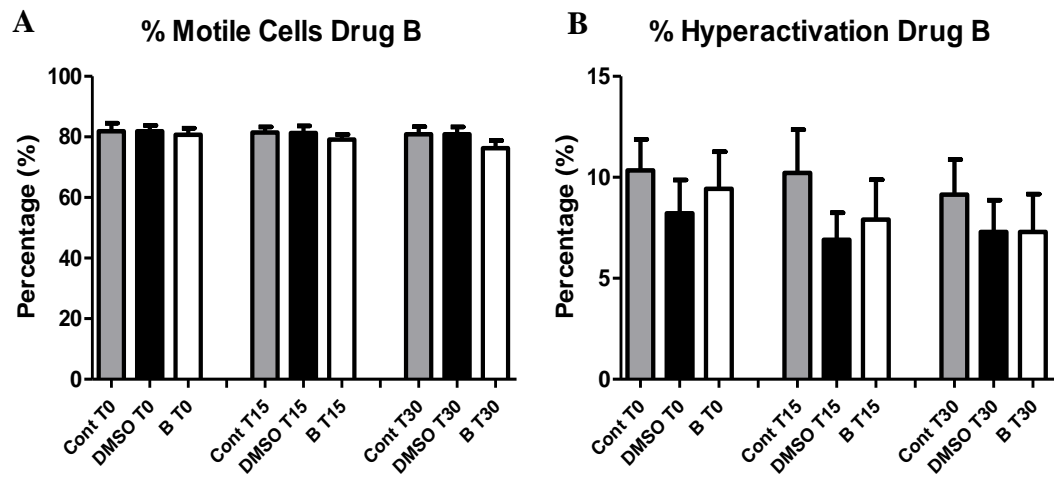
### 3.3.1 80% Motility 30 $\mu$ M Compounds A-J

#### Compound A 30 $\mu$ M 80%



**Figure 3.1: 80% sperm cell fraction motility screen using 30 $\mu$ M drug A.** No. of donors = 15. Each motility parameter was compared using ANOVA of control, DMSO and drug A groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – no significant difference. **B)** Average % Hyperactivated cells – no significant difference. Error bars indicate standard error of the mean (SEM).

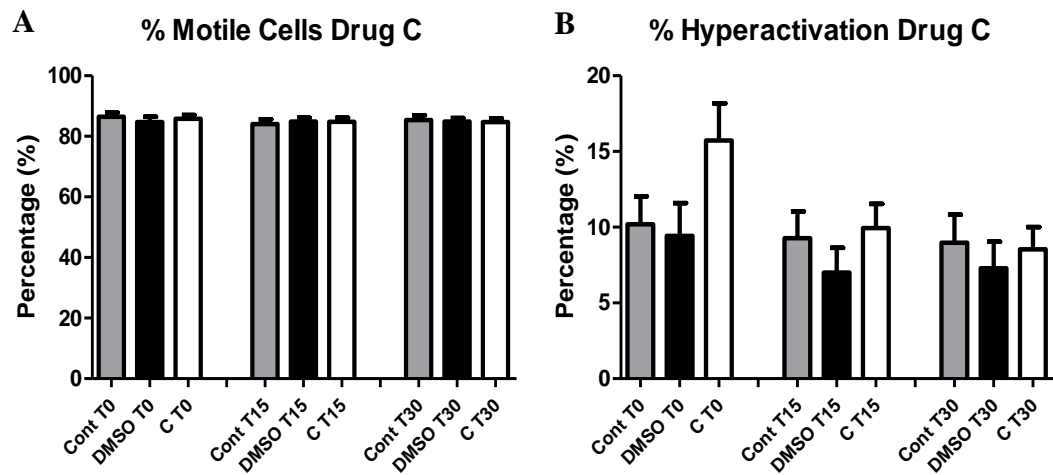
Compound B 30 $\mu$ M 80%



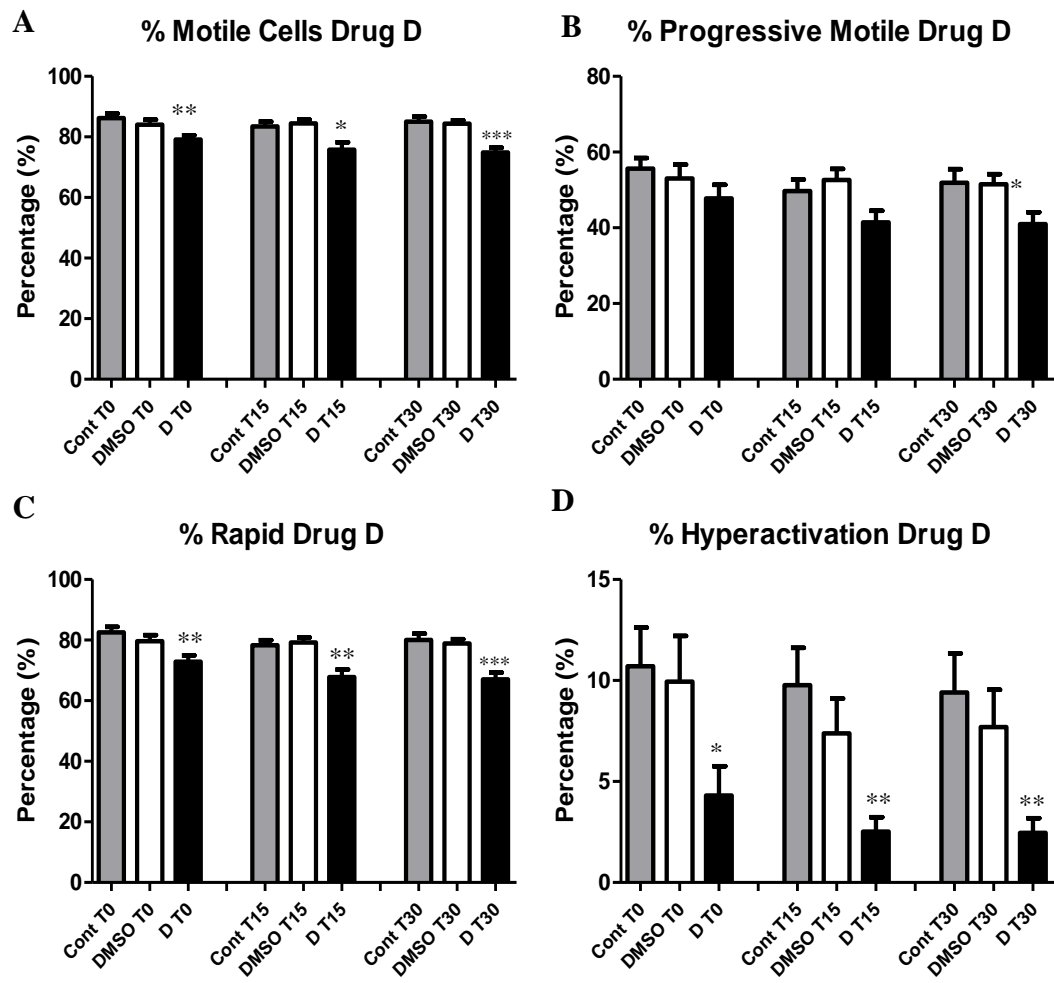
**Figure 3.2: 80% sperm cell fraction motility screen using 30 $\mu$ M drug B.** No. of donors = 14. Each motility parameter was compared using ANOVA of control, DMSO and drug B groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – no significant difference. **B)** Average % Hyperactivated cells – no significant difference. Error bars indicate standard error of the mean (SEM).

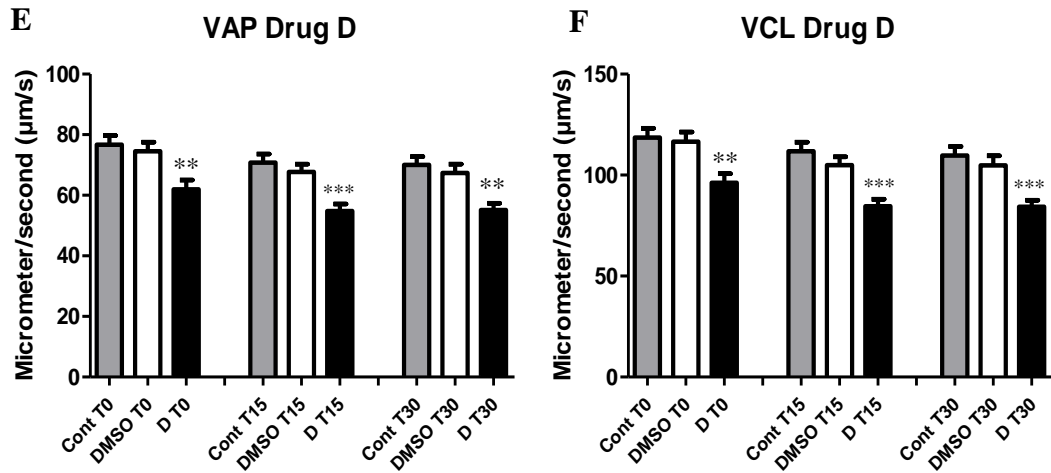


Compound C 30 $\mu$ M 80%



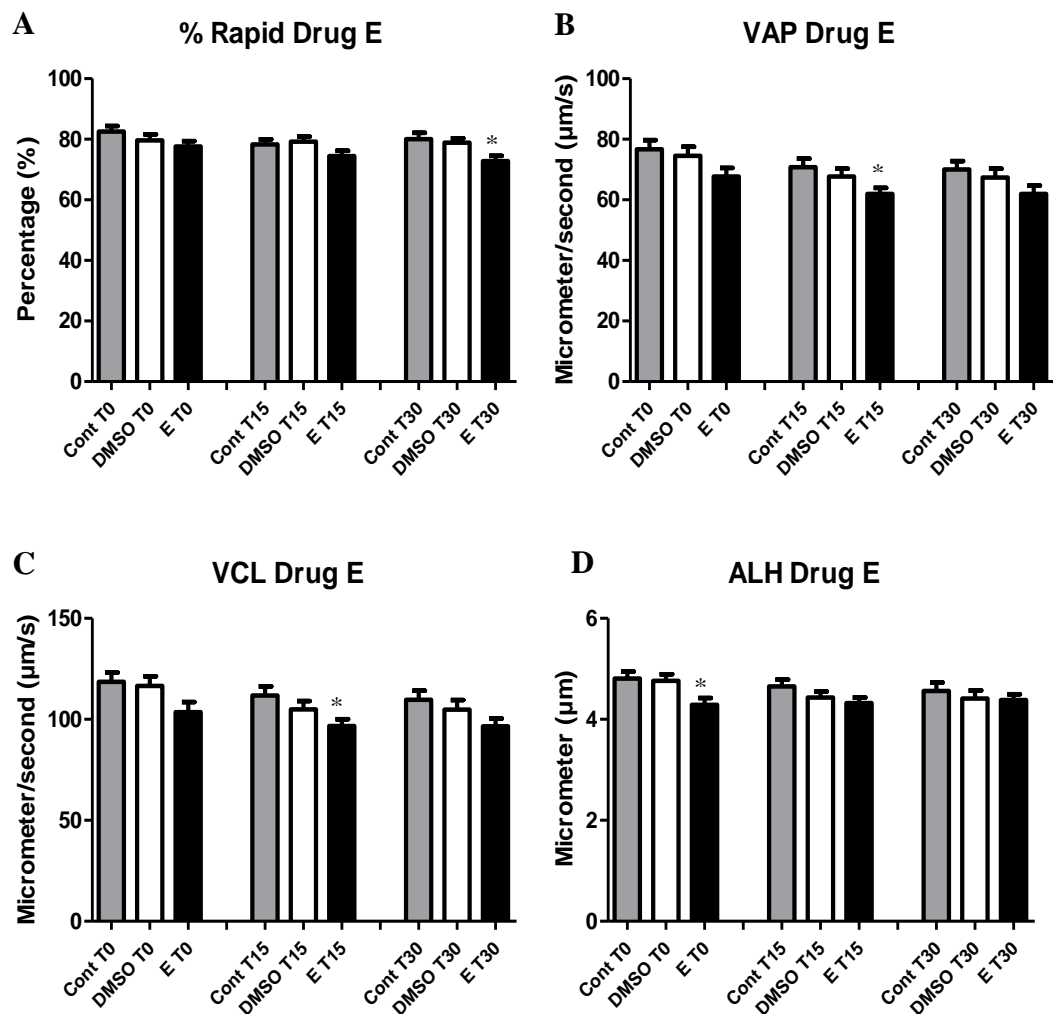
**Figure 3.3: 80% sperm cell fraction motility screen using 30 $\mu$ M drug C.** No. of donors = 14. Each motility parameter was compared using ANOVA of control, DMSO and drug C groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – no significant difference. **B)** Average % Hyperactivated cells – no significant difference. Error bars indicate standard error of the mean (SEM).

Compound D 30 $\mu$ M 80%



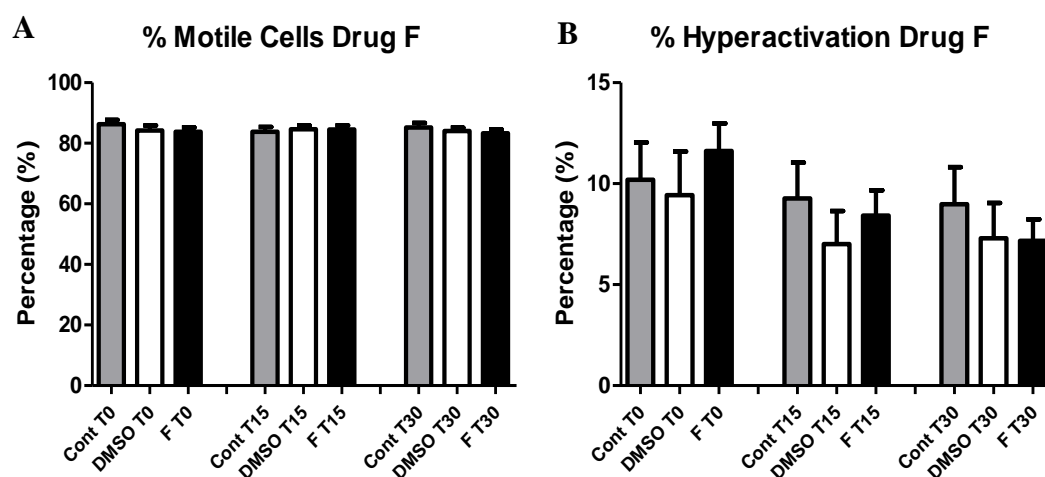
**Figure 3.4: 80% sperm cell fraction motility screen using 30 $\mu$ M drug D.** No. of donors = 13. Each motility parameter was compared using ANOVA of control, DMSO and drug D groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – T0: Control + drug D - \*\* (P<0.01). T15: Control + drug D - \* (P<0.05), DMSO + drug D - \*\* (P=0.0034). T30: Control + drug D - \*\*\* (P<0.0001), DMSO + drug D - \*\*\* (P<0.0001). **B)** Average % progressive motile cells – T0: no significant difference. T15: DMSO + drug D - \* (P=0.0351). T30: Control + drug D - \* (P=0.0211), DMSO + drug D - \* (P=0.0211). **C)** Average % rapid cells – T0: Control + drug D - \*\* (P=0.0045). T15: Control + drug D - \*\* (P<0.01), DMSO + drug D - \*\*\* (P=0.0003). T30: Control + drug D - \*\*\* (P<0.0001), DMSO + drug D - \*\*\* (P<0.0001). **D)** Average % Hyperactivated cells - T0: Control + drug D - \* (P<0.05), DMSO + drug D - \* (P<0.05). T15: Control + drug D - \*\* (P = 0.0017), DMSO + drug D - \* (P<0.05). T30: Control + drug D - \*\* (P=0.0017), DMSO + drug D - \* (P<0.05). **E)** Average VAP – T0: Control + drug D - \*\* (P=0.0028), DMSO + drug D - \* (P<0.05). T15: Control + drug D - \*\*\* (P=0.0002), DMSO + drug D - \*\* (P<0.01). T30: Control + drug D - \*\* (P<0.01), DMSO + drug D - \*\* (P<0.01). **F)** Average VCL – T0: Control + drug D - \*\* (P=0.0023), DMSO + drug D - \*\* (P=0.0023). T15: Control + drug D - \*\*\* (P<0.0001), DMSO + drug D - \*\* (P<0.01). T30: Control + drug D - \*\*\* (P=0.0003), DMSO + drug D - \*\* (P<0.01). Error bars indicate standard error of the mean (SEM).

### 80% Compound E 30 $\mu$ M

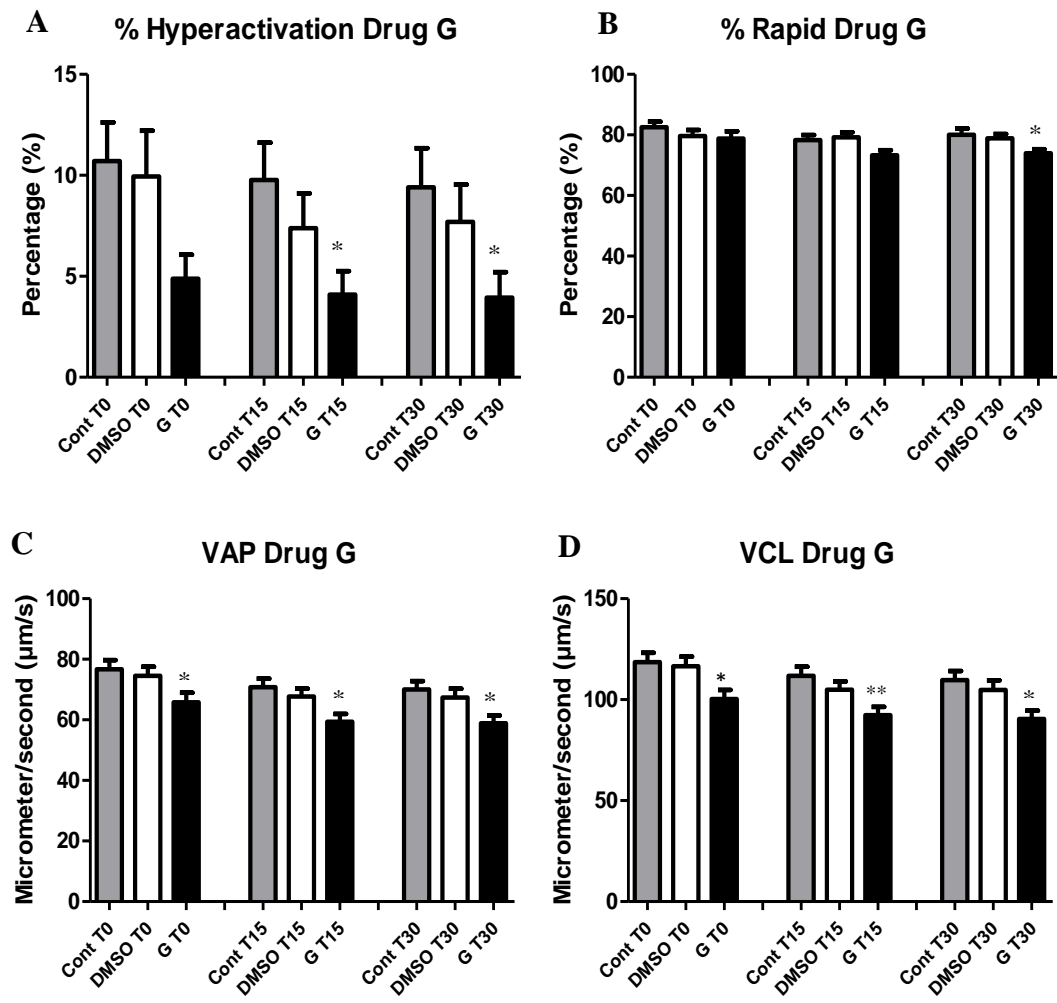


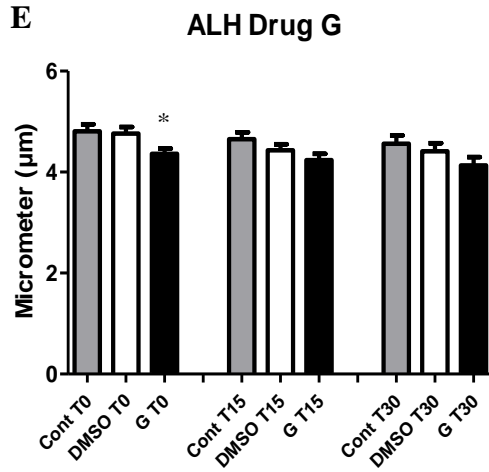
**Figure 3.5: 80% sperm cell fraction motility screen using 30 $\mu$ M drug E.** No. of donors = 13. Each motility parameter was compared using ANOVA of control, DMSO and drug E groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % rapid cells – T0+T15: no statistically significant difference. T30: Control + drug E \* (P=0.0153). **B)** Average VAP – T0: no statistically significant difference. T15: Control + drug E - \* (P<0.05). T30: no significant difference. **C)** Average VCL – T0: no statistically significant difference. T15: Control + drug E - \* (P=0.0420). T30: no statistically significant difference. **D)** Average ALH – T0: Control + drug E - \* (P=0.0173). T15+T30: no statistically significant difference. Error bars indicate standard error of the mean (SEM).

Compound F 30 $\mu$ M 80%



**Figure 3.6: 80% sperm cell fraction motility screen using 30 $\mu$ M drug F.** No. of donors = 14. Each motility parameter was compared using ANOVA of control, DMSO and drug F groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – no statistically significant difference. **B)** Average % Hyperactivated cells – no statistically significant difference. Error bars indicate standard error of the mean (SEM).

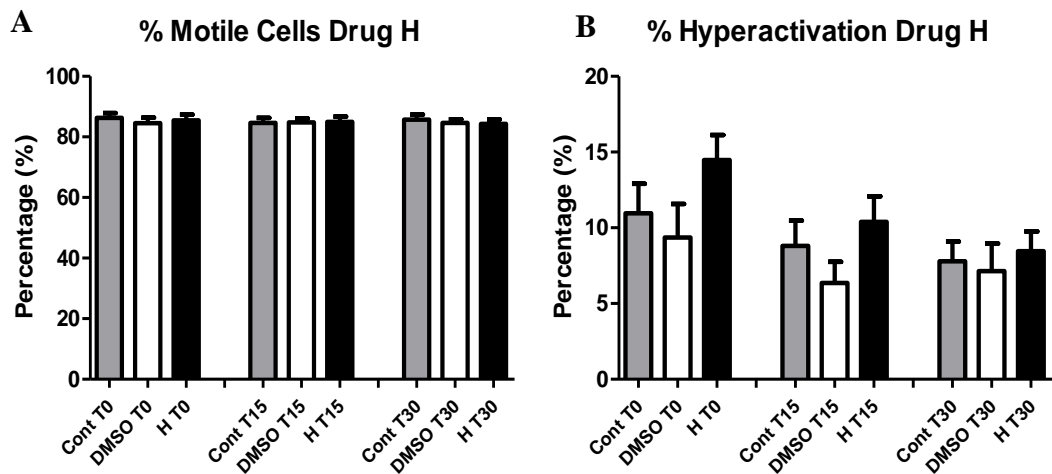
Compound G 30 $\mu$ M 80%



**Figure 3.7: 80% sperm cell fraction motility screen using 30µM drug G.** No. of donors = 13.

Each motility parameter was compared using ANOVA of control, DMSO and drug G groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % Hyperactivated cells – T0: no statistically significant difference. T15: Control + drug G - \* (P=0.0422). T30: Control + drug G - \* (P=0.0324). **B)** Average % rapid cells – T0: no statistically significant difference. T15: DMSO + drug G - \* (P=0.0362). T30: Control + drug G - \* (P=0.0337). **C)** Average VAP – T0: Control + drug G - \* (P=0.0378). T15: Control + drug G - \* (P=0.0130). T30: Control + drug G - \* (P=0.0186). **D)** Average VCL – T0: Control + drug G - \* (P=0.0150), DMSO + drug G - \* (P=0.0150). T15: Control + drug G - \*\* (P=0.0094). T30: Control + drug G - \* (P=0.0117). **E)** Average ALH – T0: Control + drug G - \* (P=0.0324). T15 + T30: no statistically significant difference. Error bars indicate standard error of the mean (SEM).

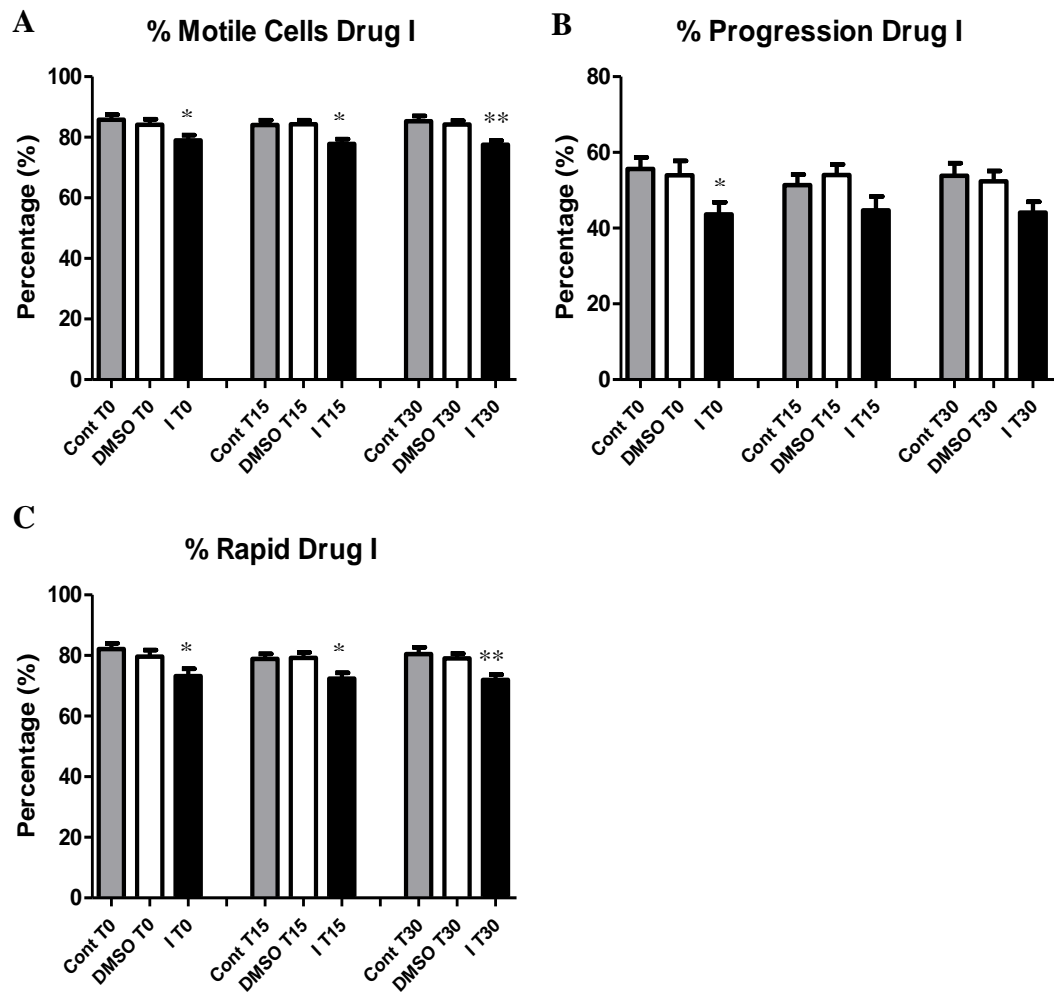
Compound H 30 $\mu$ M 80%



**Figure 3.8: 80% sperm cell fraction motility screen using 30 $\mu$ M drug H.** No. of donors = 13. Each motility parameter was compared using ANOVA of control, DMSO and drug H groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – no statistically significant difference. **B)** Average % Hyperactivated cells – no statistically significant difference. Error bars indicate standard error of the mean (SEM).

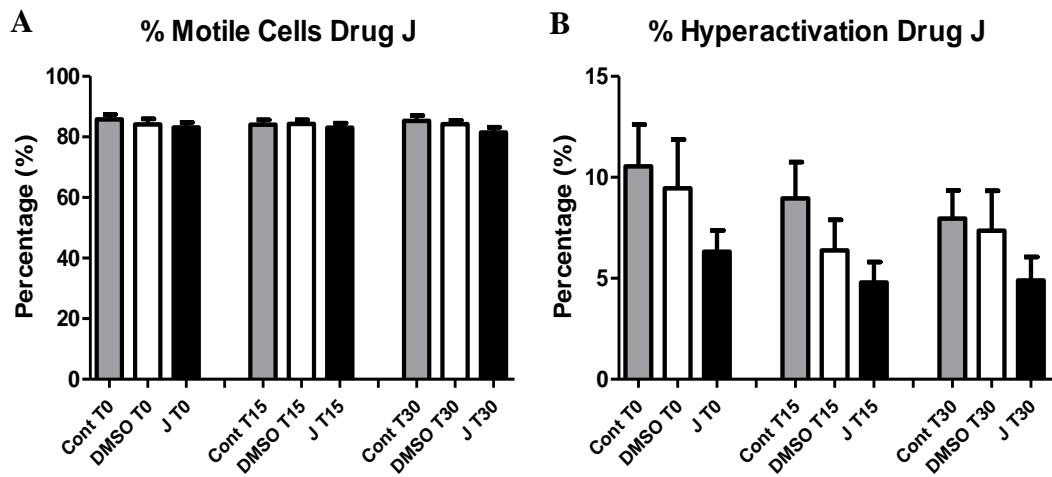


Compound I 30 $\mu$ M 80%



**Figure 3.9: 80% sperm cell fraction motility screen using 30 $\mu$ M drug I.** No. of donors = 12. Each motility parameter was compared using ANOVA of control, DMSO and drug I groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – T0: Control + drug I - \* (P=0.0262). T15: Control + drug I - \* (P<0.05), DMSO + drug I - \* (P<0.05). T30: Control + drug I - \*\* (P=0.0012), DMSO + drug I - \*\* (P=0.0012). **B)** Average % progressive cells – T0: Control + drug I - \* (P=0.0357). T15+T30: no statistically significant difference. **C)** Average % rapid cells – T0: Control + drug I - \* (P=0.0207), T15: Control + drug I - \* (P=0.0170), DMSO and drug I - \* (P=0.0170). T30: Control + drug I - \*\* (P=0.0053). Error bars indicate standard error of the mean (SEM).

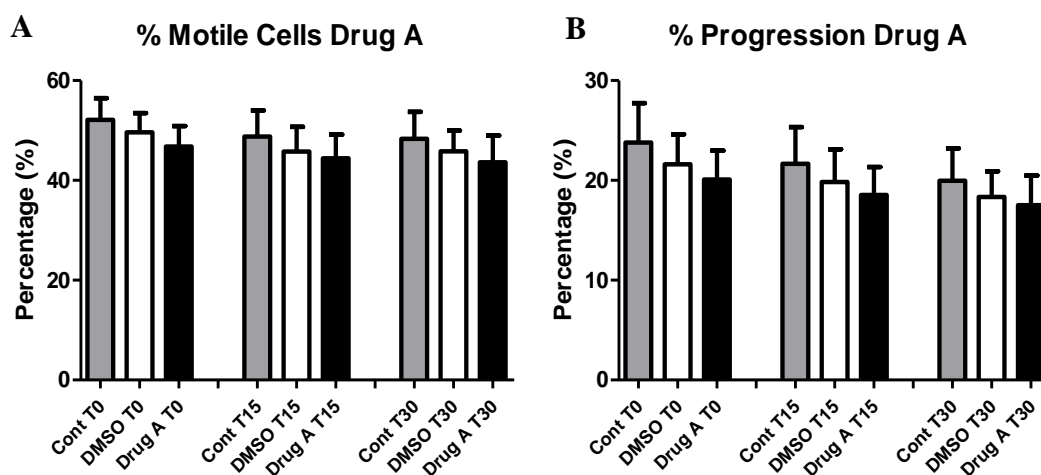
Compound J 30 $\mu$ M 80%



**Figure 3.10: 80% sperm cell fraction motility screen using 30 $\mu$ M drug J.** No. of donors = 12. Each motility parameter was compared using ANOVA of control, DMSO and drug J groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – no statistically significant difference. **B)** Average % Hyperactivated cells – no statistically significant difference. Error bars indicate standard error of the mean (SEM).

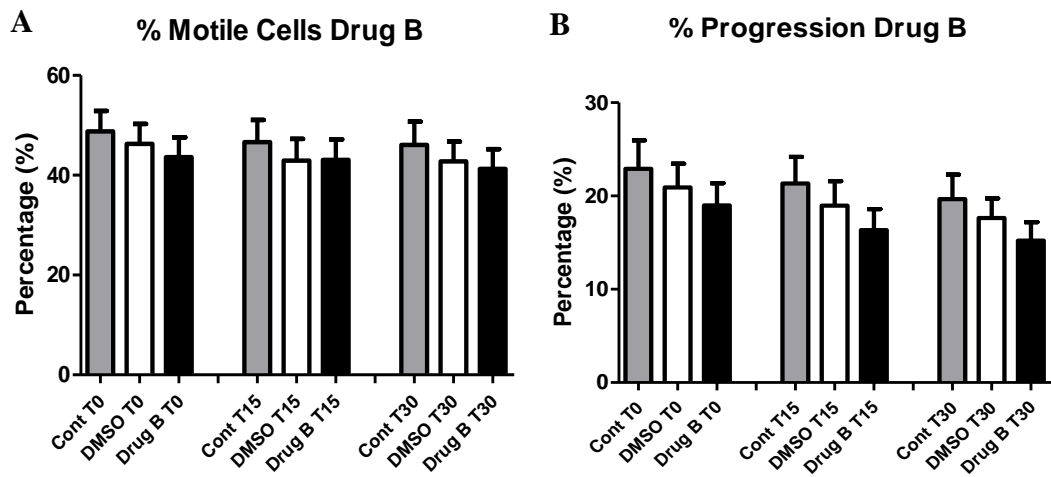
### 3.3.2 40% Motility 30 $\mu$ M Compounds A-J

#### Compound A 30 $\mu$ M 40%



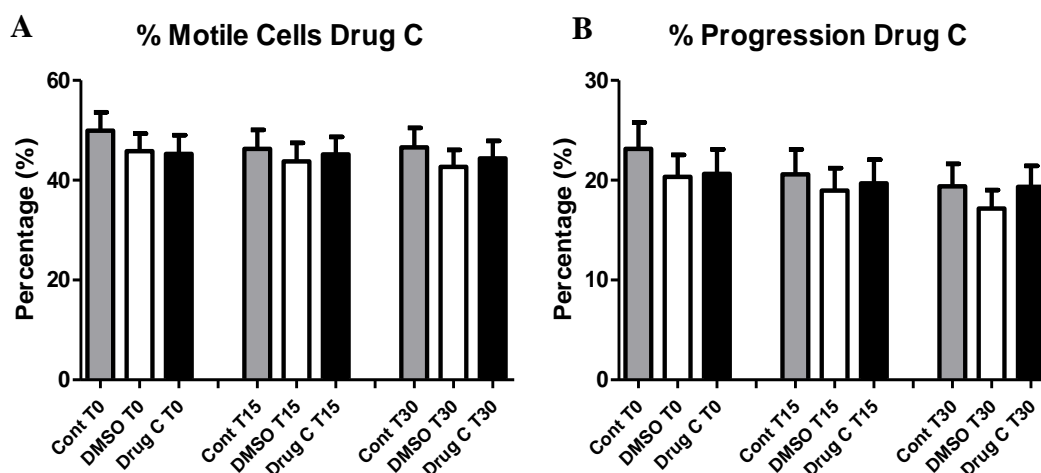
**Figure 3.11: 40% sperm cell fraction motility screen using 30 $\mu$ M drug A.** No. of donors = 9. Each motility parameter was compared using ANOVA of control, DMSO and drug A groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – no statistically significant difference. **B)** Average % progressive cells – no statistically significant difference. Error bars indicate standard error of the mean (SEM).

Compound B 30 $\mu$ M 40%



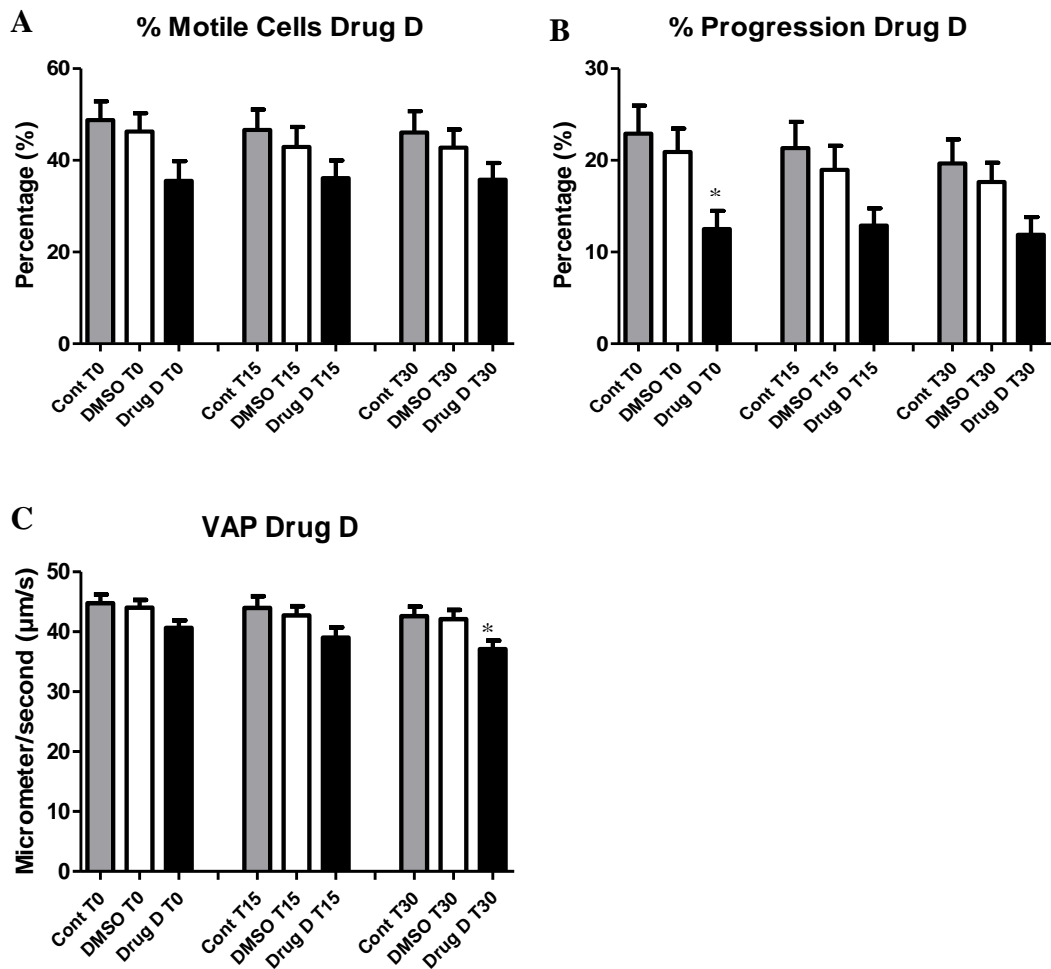
**Figure 3.12: 40% sperm cell fraction motility screen using 30 $\mu$ M drug B.** No. of donors = 12. Each motility parameter was compared using ANOVA of control, DMSO and drug B groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – no statistically significant difference. **B)** Average % progressive cells – no statistically significant difference. Error bars indicate standard error of the mean (SEM).

Compound C 30 $\mu$ M 40%



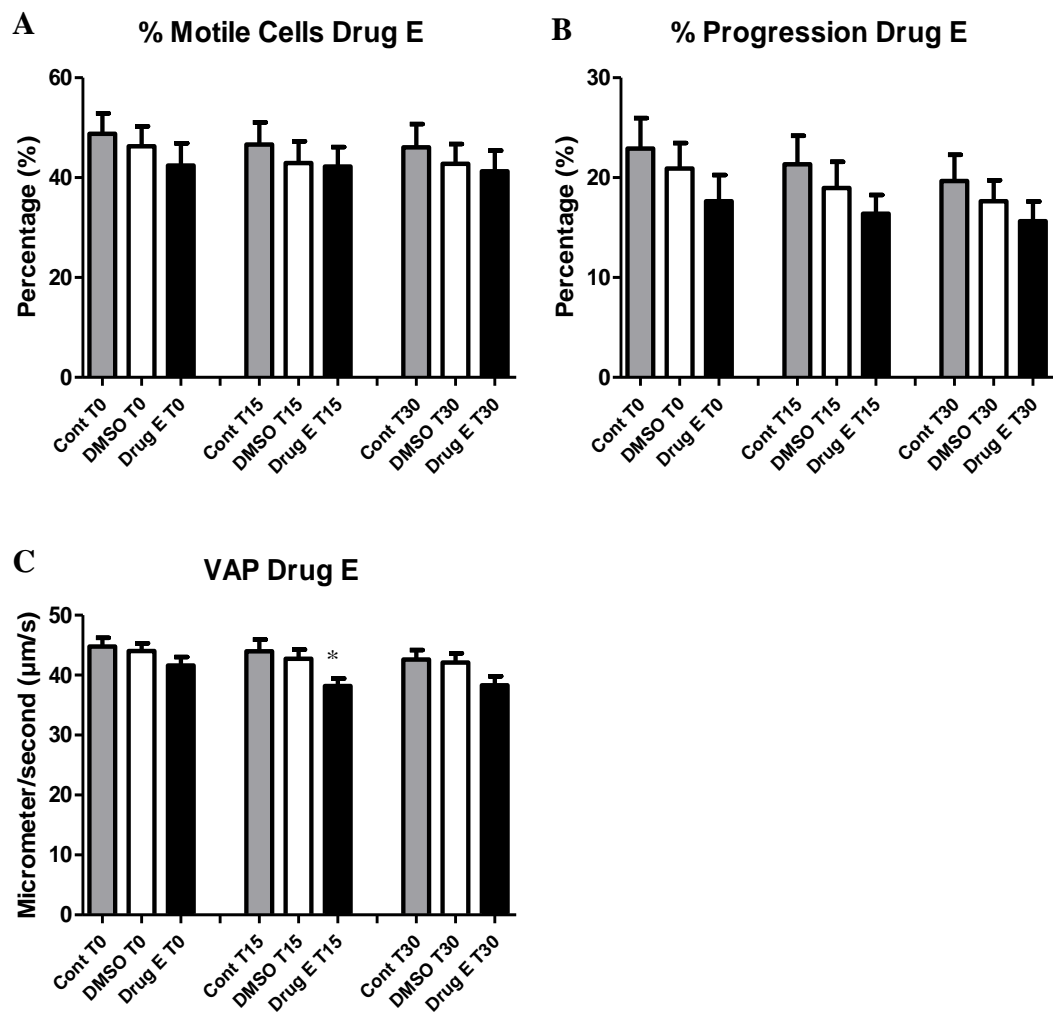
**Figure 3.13: 40% sperm cell fraction motility screen using 30 $\mu$ M drug C.** No. of donors = 14. Each motility parameter was compared using ANOVA of control, DMSO and drug C groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – no statistically significant difference. **B)** Average % progressive cells – no statistically significant difference. Error bars indicate standard error of the mean (SEM).

### Compound D 40 $\mu$ M 40%



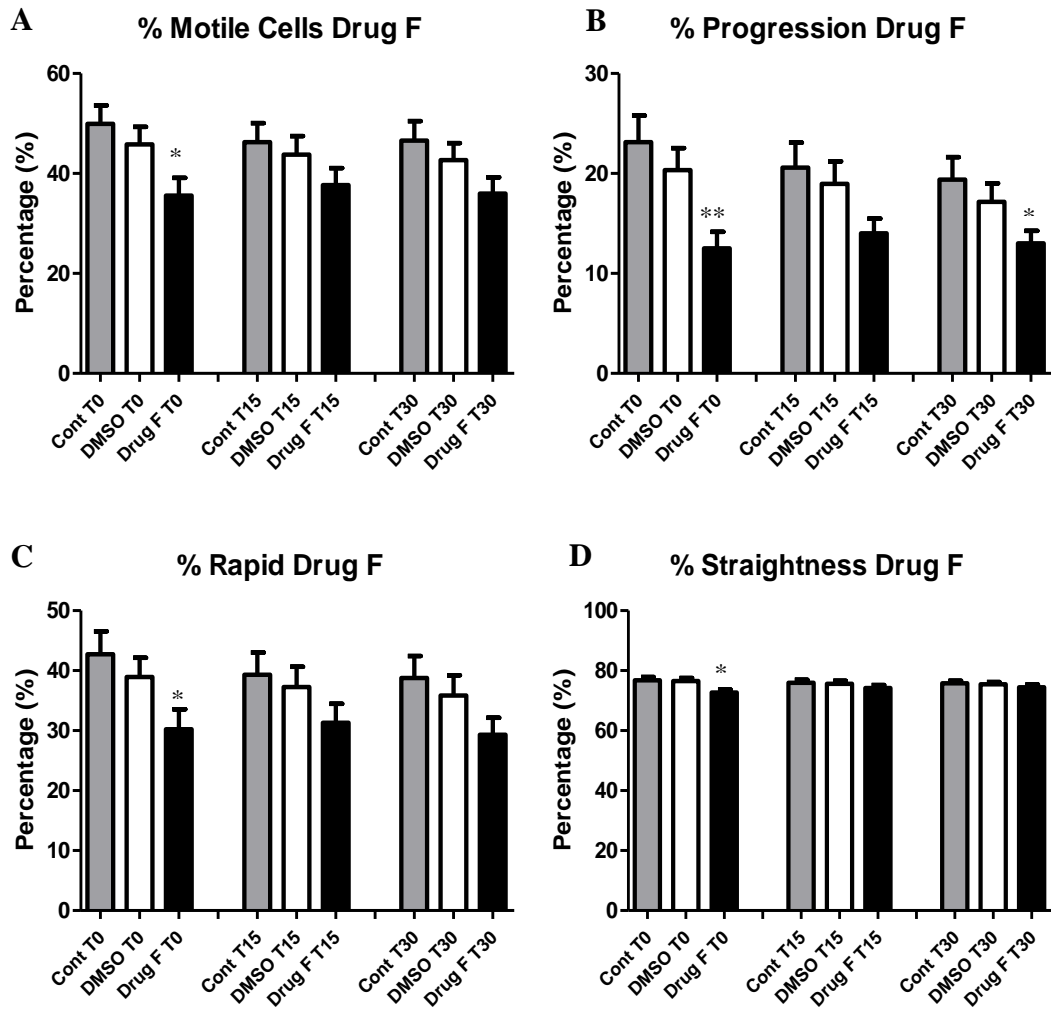
**Figure 3.14: 40% sperm cell fraction motility screen using 30 $\mu$ M drug D.** No. of donors = 12. Each motility parameter was compared using ANOVA of control, DMSO and drug D groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – no statistically significant difference. **B)** Average % progressive cells – T0: Control + drug D - \* (P=0.0166). T15+T30: no statistically significant difference. **C)** Average VAP – T0+T15: no statistically significant difference. T30: Control + drug D - \* (P=0.027). Error bars indicate standard error of the mean (SEM).

Compound E 30 $\mu$ M 40%



**Figure 3.15: 40% sperm cell fraction motility screen using 30 $\mu$ M drug E.** No. of donors = 12. Each motility parameter was compared using ANOVA of control, DMSO and drug E groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – no statistically significant difference was seen. **B)** Average % progressive cells – no statistically significant difference. **C)** Average VAP – T0: no statistically significant difference. T15: Control + drug E - \* (P=0.0395). T30: no statistically significant difference. Error bars indicate standard error of the mean (SEM).

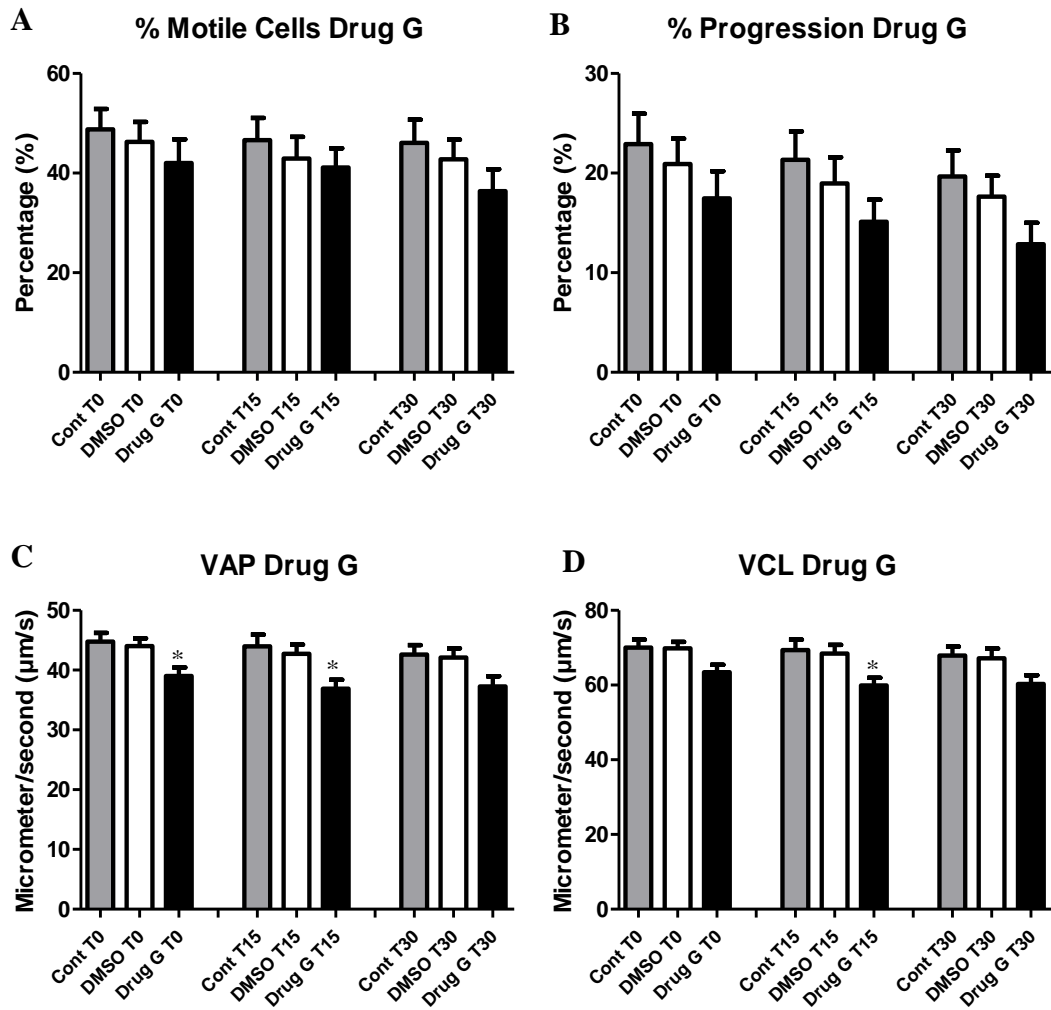
Compound F 30 $\mu$ M 40%



**Figure 3.16: 40% sperm cell fraction motility screen using 30 $\mu$ M drug F.** No. of donors = 14. Each motility parameter was compared using ANOVA of control, DMSO and drug F groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – T0: Control + drug F - \* (P=0.0434). T15+T30: no statistically significant difference. **B)** Average % progressive cells – T0: Control + drug F - \*\* (P=0.0045), DMSO + drug F - \* (P<0.05). T15: no statistically significant difference. T30: Control + drug F - \* (P<0.05). **C)** Average % rapid cells – T0: Control + drug F - \* (P=0.0209). T15+T30: no statistically significant difference. **D)** Average % straightness – T0: Control + drug F - \* (P=0.0132), DMSO + drug F - \* (P=0.0132). T15+T30: no statistically significant difference. Error bars indicate standard error of the mean (SEM).

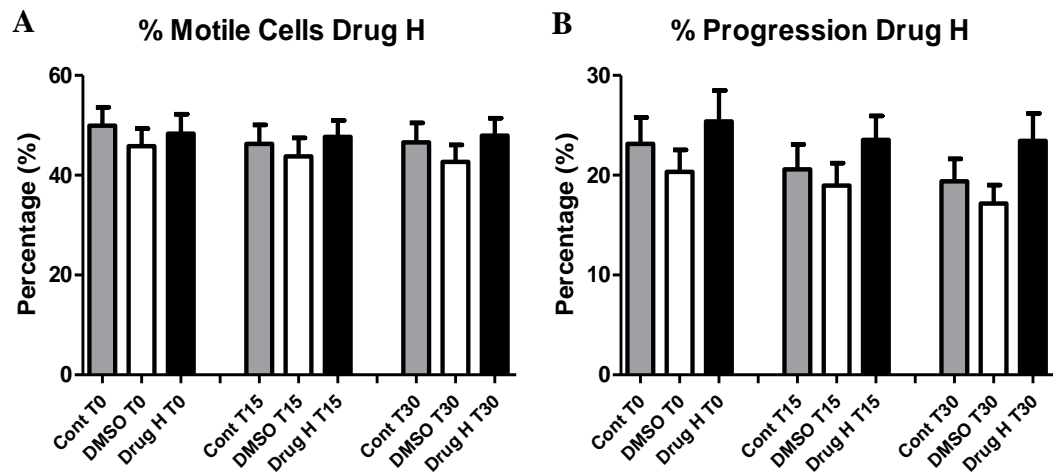


Compound G 30 $\mu$ M 40%



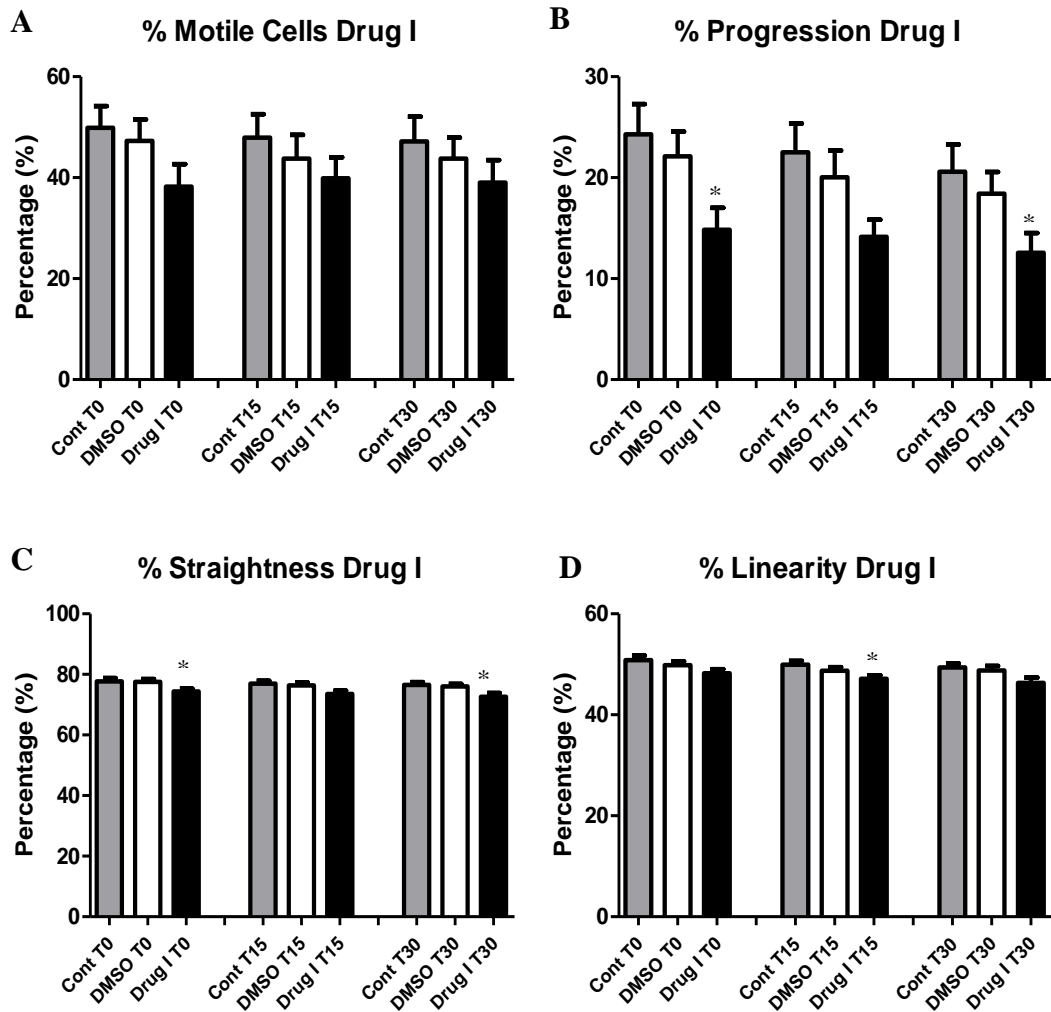
**Figure 3.17: 40% sperm cell fraction motility screen using 30 $\mu$ M drug G.** No. of donors = 12. Each motility parameter was compared using ANOVA of control, DMSO and drug G groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – no statistically significant difference. **B)** Average % progressive cells – no statistically significant difference. **C)** Average VAP – T0: Control + drug G - \* (P=0.0130), DMSO + drug G - \* (P=0.0130). T15: Control + drug G - \* (P=0.0115), DMSO + drug G - \* (P=0.0115). T30: no statistically significant difference. **D)** Average VCL – T0: no statistically significant difference. T15: Control + drug G - \* (P=0.0161), DMSO + drug G - \* (P=0.0161). T30: no statistically significant difference. Error bars indicate standard error of the mean (SEM).

Compound H 30 $\mu$ M 40%



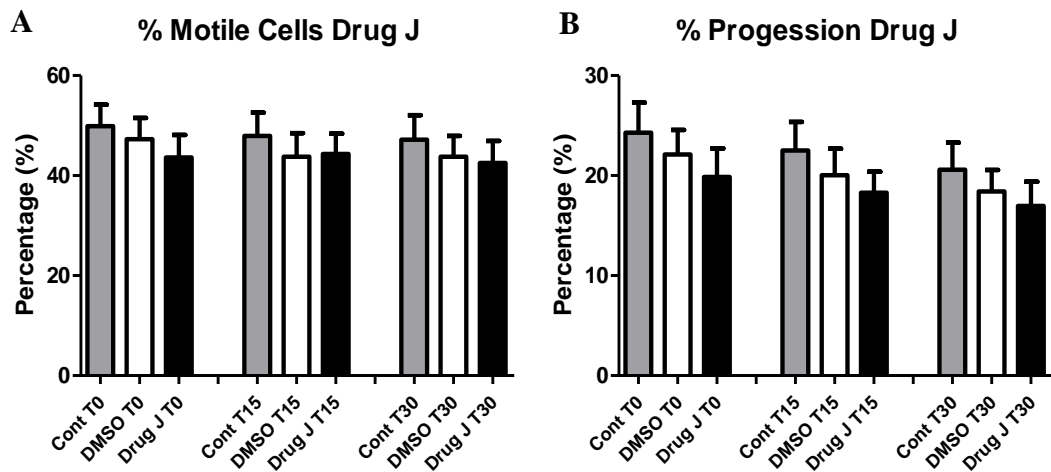
**Figure 3.18: 40% sperm cell fraction motility screen using 30 $\mu$ M drug H.** No. of donors = 14. Each motility parameter was compared using ANOVA of control, DMSO and drug H groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – no statistically significant difference. **B)** Average % progressive cells – no statistically significant difference. Error bars indicate standard error of the mean (SEM).

Compound I 30 $\mu$ M 40%



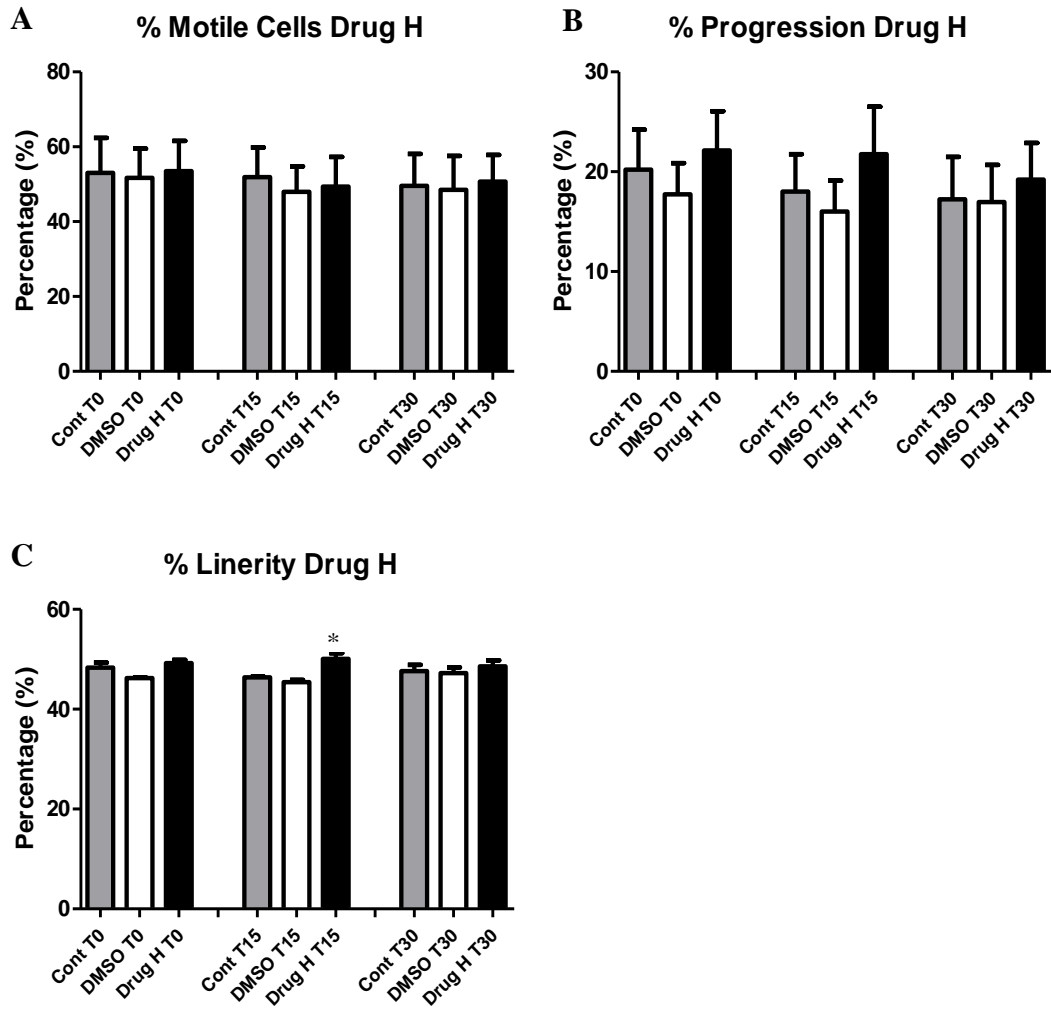
**Figure 3.19: 40% sperm cell fraction motility screen using 30 $\mu$ M drug I.** No. of donors = 11. Each motility parameter was compared using ANOVA of control, DMSO and drug I groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – no statistically significant difference. **B)** Average % progressive cells – T0: Control + drug I - \* (P=0.0355). T15: no statistically significant difference. T30: Control + drug I - \* (P<0.05). **C)** Average % straightness – T0: Control + drug I - \* (P=0.0318). T15: no statistically significant difference. T30: Control + drug I - \* (P=0.0194). **D)** Average % linearity – T0: no statistically significant difference. T15: Control + drug I - \* (P=0.0335). T30: no statistically significant difference. Error bars indicate standard error of the mean (SEM).

Compound J 30 $\mu$ M 40%



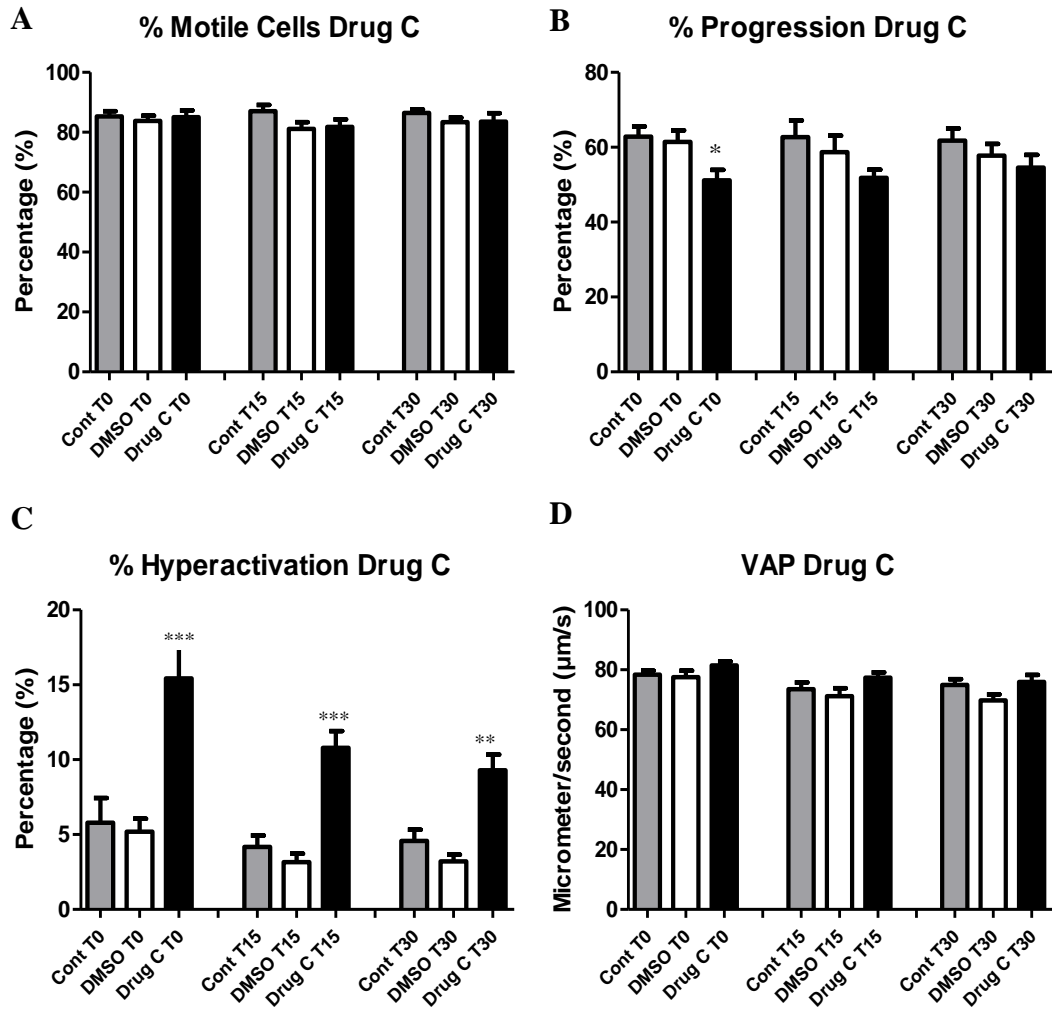
**Figure 3.20: 40% sperm cell fraction motility screen using 30 $\mu$ M drug J.** No. of donors = 11. Each motility parameter was compared using ANOVA of control, DMSO and drug J groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – no statistically significant difference. **B)** Average % progressive cells – no statistically significant difference. Error bars indicate standard error of the mean (SEM).

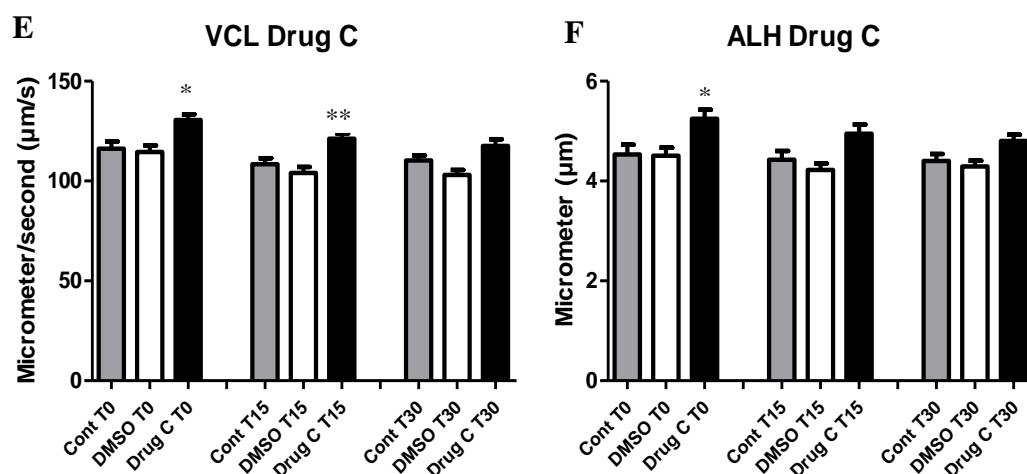
### 3.3.3 40% 10 $\mu$ M Compound H



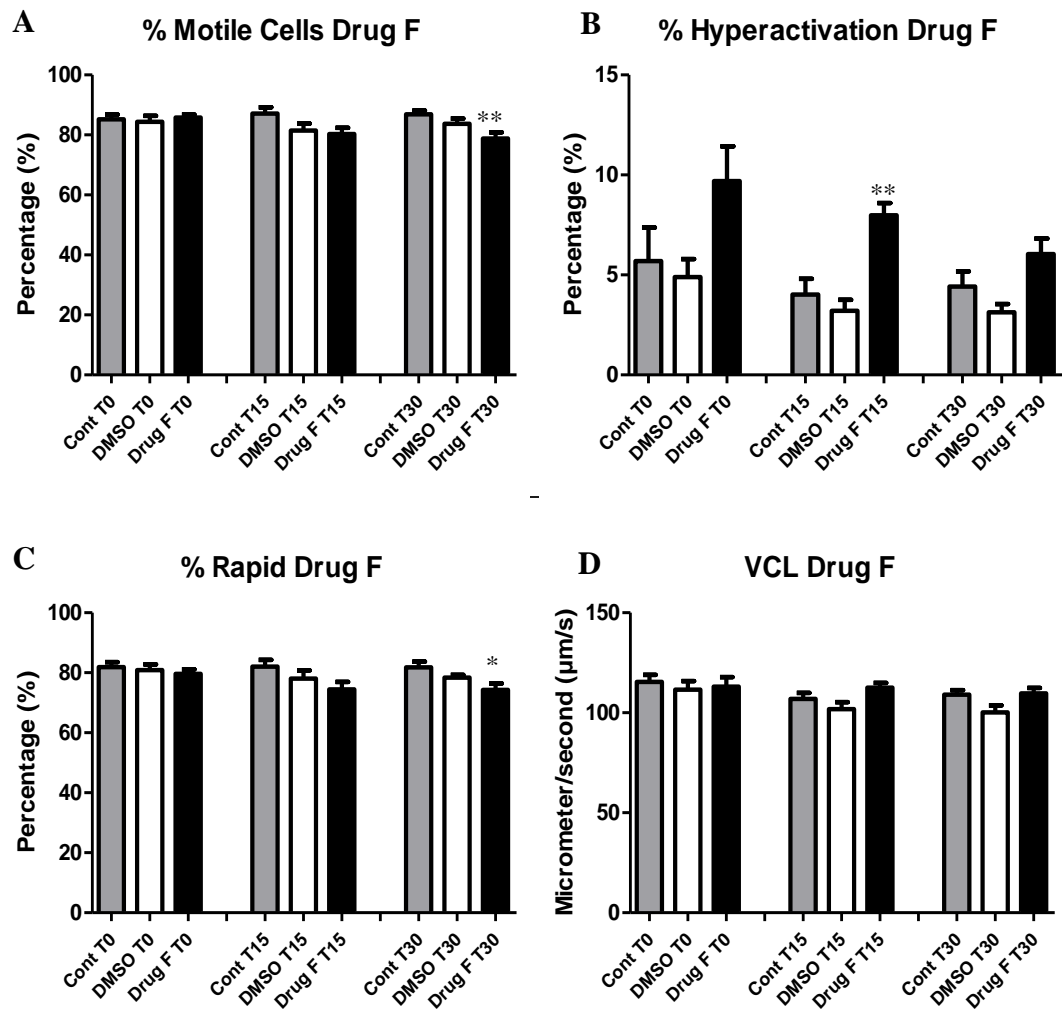
**Figure 3.21: 40% sperm cell fraction motility screen using 10 $\mu$ M drug H.** N = 4. Each motility parameter was compared using ANOVA of control, DMSO and drug H groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – no statistically significant difference. **B)** Average % progressive cells – no statistically significant difference. **C)** Average % linearity – T0: DMSO + drug H - \* (P=0.0302). T15: Control + drug H - \* (P<0.05), DMSO + drug H - \*\* (P=0.0038). T30: no statistically significant difference. Error bars indicate standard error of the mean (SEM).

3.3.4 80% 100 $\mu$ M - Selected compounds  
 Compound C 100 $\mu$ M 80%

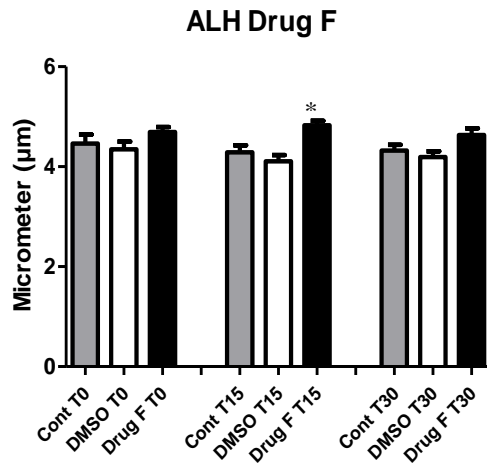




**Figure 3.22: 80% sperm cell fraction motility screen using 100μM drug C.** N = 8. Each motility parameter was compared using ANOVA of control, DMSO and drug C groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – no statistically significant difference. **B)** Average % progressive cells – T0: Control + drug C - \* (P=0.0173), DMSO + drug C - \* (P=0.0173). T15+T30: no statistically significant difference. **C)** Average % Hyperactivated cells – T0: Control + drug C - \*\*\* (P=0.0003), DMSO + drug C - \*\*\* (P=0.0003). T15: Control + drug C - \*\*\* (P<0.0001), DMSO + drug C (P<0.0001). T30: Control + drug C - \*\* (P<0.01), DMSO + drug C - \*\*\* (P<0.0001). **D)** Average VAP – no statistically significant difference. **E)** Average VCL – T0: Control + drug C - \* (P<0.05), DMSO + drug C - \*\* (P=0.0039). T15: Control + drug C - \*\* (P<0.01), DMSO + drug C - \*\*\* (P=0.0006). T30: DMSO + drug C - \*\* (P=0.0054). **F)** Average ALH – T0: Control + drug C - \* (P=0.0116), DMSO + drug C - \* (P=0.0116). T15: DMSO + drug C - \* (P=0.0150). T30: DMSO + drug C - \* (P=0.0241). Error bars indicate standard error of the mean (SEM).

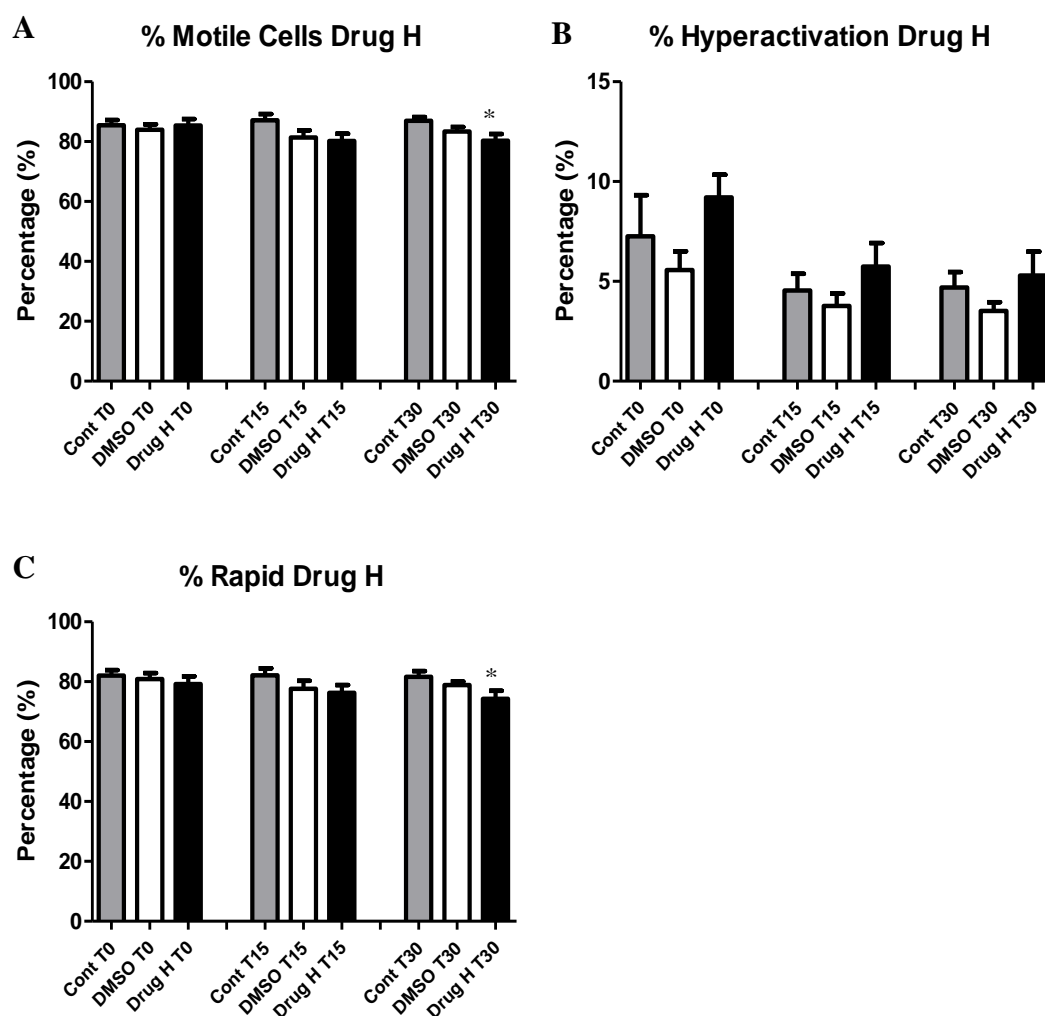
Compound F 100 $\mu$ M 80%





**Figure 3.23: 80% sperm cell fraction motility screen using 100µM drug F.** N = 8. Each motility parameter was compared using ANOVA of control, DMSO and drug F groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – T0+T15: no statistically significant difference. T30: Control + drug F - \*\* (P<0.01). **B)** Average % Hyperactivated cells – T0: no statistically significant difference. T15: Control + drug F - \*\* (P<0.01), DMSO + drug F - \*\*\* (P<0.0001). T30: DMSO + drug F - \* (P=0.0199). **C)** Average % rapid cells – T0+T15: no statistically significant difference. T30: Control + drug F - \* (P<0.05). **D)** Average VCL of cells – T0: no statistically significant difference. T15: DMSO + drug F - \* (P=0.0595). T30: no statistically significant difference. **E)** Average ALH of cells – T0: no statistically significant difference. T15: Control + drug F - \* (P<0.05), DMSO + drug F - \*\* (P=0.0011). T30: DMSO + drug F - \* (P=0.0516). Error bars indicate standard error of the mean (SEM).

Compound H 100 $\mu$ M 80%



**Figure 3.24: 80% sperm cell fraction motility screen using 100 $\mu$ M drug H.** N = 8. Each motility parameter was compared using ANOVA of control, DMSO and drug H groups at Time 0 mins (T0), Time 15 mins (T15) and Time 30 mins (T30). **A)** Average % motile cells – T0+T15: no statistically significant difference. T30: Control + drug H - \* (P=0.0403). **B)** Average % HA cells – no statistically significant difference. **C)** Average % rapid cells – T0+T15: no statistically significant difference. T30: Control + drug H - \* (P=0.051). Error bars indicate standard error of the mean (SEM).

### 3.4 Discussion

A series of motility experiments were undertaken investigating 10 compounds from the UoD DDU that were previously identified to elicit  $[Ca^{2+}]_i$  responses in human spermatozoa using high-throughput Flex Station screening. These compounds were analysed at a final concentration of 30 $\mu$ M using two cell populations (40% and 80% spermatozoa fractions) derived from healthy donors, to determine if they induced an increase in specific motility parameters. Compounds that elicited an effect on motility parameters – C and F - were subsequently screened at 10 $\mu$ M and 100 $\mu$ M concentrations to determine if this enhanced the effects observed.

Compound	Name	Target
A	Buspirone	5-HT and Dopamine type 2 (DA2) receptor antagonist. Used to treat anxiety disorders.
B	Clozapine	Antagonistic effects at D2 receptors in the mesolimbic pathway and 5-HT <sub>2A</sub> receptors. Anti-psychotic, treats schizophrenia.
C	Primaquine	Anti-malarial agent – mechanism not known
D	Trifluoperazine	Blocks postsynaptic mesolimbic dopaminergic D1 and D2 receptors in the brain. Anti-psychotic, treats symptoms of schizophrenia and anxiety.
E	Naftopidil	Selective $\alpha_1$ -adrenergic receptor antagonist. Anti-hypertensive drug.
F	Diphenoxylate	Used as an antidiarrheal, usually in combination with atropine. Opioid receptor agonist.

G	MK886	Antagonist of 5-lipoxygenase activating protein. Helps in treating atherosclerosis.
H	Iloperidone	Dopamine D2 and 5-HT <sub>2A</sub> receptor antagonist. Anti-psychotic for schizophrenia symptoms.
I	Nebivolol	$\beta$ <sub>1</sub> -receptor antagonist. Used in treatment of hypertension.
J	Gepirone	Selective partial agonist of the 5-HT <sub>1A</sub> receptor. Under clinical development as an antidepressant and anxiolytic.

**Table 3.1:** List of the 10 compounds from the UoD DDU chemogenomics library which all caused an increase in intracellular calcium concentration in spermatozoa seen during the Flex Station high-throughput screen. This study was blind so compounds were referred to as A-J. Their respective names are given above along with their mechanism of action and medical condition they are prescribed for.

#### 3.4.1 Mechanism of action of compounds that induce a motility enhancing effect

Two of the compounds - drug C and F - that indicated positive effects on spermatozoa motility, are both unique compounds with very different mechanisms of action.

Drug F is an opioid receptor agonist known as Diphenoxylate. Endogenous opioids are neurotransmitters which act in the central and peripheral nervous system (Elde R, & Hökfelt T, 1993). They are located in various places in the body; adrenal medulla, pancreatic islets, pituitary, intestinal and bronchial mucosa (Bostwick et al., 1987) and also in male reproductive organs (Fabbri et al., 1986; Foresta et al., 1986; Kew et al., 1990). Opioids are thought to inhibit neuronal excitability via two mechanisms; the inhibition of calcium and the enhancement of potassium conductance (Bean, 1989; North RA, 1993).

Opioid receptors are 7 Transmembrane G-protein coupled receptors (TM GPCRs) (Childers, 1991), of which there are three types; mu, delta and kappa opioid receptors (MOR, DOR and KOR) (McNally & Akil, 2002). Previous research has indicated the expression of these receptors in human spermatozoa (Agirregoitia et al., 2006) and suggested that the opioid system is involved in the control of spermatozoa motility. Various studies have observed reduced motility (asthenozoospermia) after *in-vitro* and *in-vivo* exposure to opioid receptor agonists. The MOR agonist morphine causes reduced numbers of grade a and b sperm at low concentrations (0.1  $\mu$ M) after a longer period of time (Agirregoitia et al., 2006). Naltrindole, a DOR antagonist, decreases the percentage of sperm a and b grades immediately after exposure *in-vitro* (1mM), but this decrease in spermatozoa motility weakens over time (Agirregoitia et al., 2006).

Heroin and methadone addicts (MOR action) show a spermatozoa pathology of decreased motility (Ragni et al., 1988). More recently, a study demonstrated a correlation between opiate consumption, impaired spermatozoa motility parameters and increased chromatin damage. The group of men with the highest intake of opiate consumption showed the poorest semen motility parameters and highest % DNA fragmentation index (Safarinejad et al., 2013). Chronic heroin addiction has also been linked to oxidative stress (Agirregoitia et al., 2006), a significant factor in the aetiology of male infertility that can lead to increased DNA fragmentation (Kodama et al., 1977; Pasqualotto et al., 2000).

Previous studies indicated that horse DOR is involved in motility and capacitation of spermatozoa, which are both calcium dependant events. The use of opioid antagonist, naloxone, caused a dose dependent decrease in the percent of progressive cells. Low concentrations (10mM) significantly reduced spermatozoa progressive motility and velocity; whereas high concentrations (1nM) show the opposite effect. Additionally, low concentrations negatively affect capacitation and viability while the opposite effect is

induced by high concentrations. These effects were seen after 180 minutes (Albrizio, et al., 2005).

Diphenoxylate (drug F) is an opioid agonist analgesic, specifically a MOR agonist, with an optimum binding affinity ( $K_i$ ) of 12.37nM (Volpe et al., 2011). The activation of MOR results in the inhibition of adenylyl cyclase and calcium channel conductance via pertussis toxin-sensitive  $G_i/G_o$  proteins, and stimulation of inwardly rectifying potassium channels (Liu & Anand, 2001). This stimulation, and the fact that opiates are known to enhance potassium conductance, could explain the increase in % HA seen after 15 minutes of incubation with drug F at 100 $\mu$ M. HA, among other spermatozoa physiological processes, are controlled by changes in intracellular pH, membrane voltage and  $[Ca^{2+}]_i$  concentration (Ho and Suarez, 2001; Publicover, et al., 2008). Cellular responses in spermatozoa are controlled by a unique set of ion channels (Darszon et al., 2011; Santi et al., 2013). Under normal conditions, mouse and human spermatozoa have negative resting membrane potentials that are dependent on the activity of  $K^+$  channels, and the magnitude of this channel exerts a strong influence over  $Ca^{2+}$  influx as it determines the gating of CatSper and sets the driving force for  $Ca^{2+}$  entry through these channels (Mansell et al., 2014). Recent studies on human spermatozoa illustrate that  $K^+$  conductance is enhanced by high  $[Ca^{2+}]_i$  (Mannowetz, et al., 2013). Inward rectifying potassium channels are found on spermatozoa membranes and activation induces HA by influx of  $Ca^{2+}$  ions. This explains the increase in  $[Ca^{2+}]_i$  seen in the Flex-Station screen and the initial increase in % HA after exposure of spermatozoa to Diphenoxylate. The activation of MOR channels also inhibits adenylyl cyclase, which may take time to come into effect, explaining the significant decrease in % motility and % rapid cells by T30. After the initial activation of inward rectifying  $K^+$  channels, this rapid motility could not be maintained, using up the cellular cAMP, and

the inhibition of the AC enzyme meant no more cAMP was produced therefore having detrimental effects on spermatozoa motility.

Drug C is an anti-malarial known as primaquine and little is known about its mechanism of action within the human body. Primaquine is the only antimalarial that is active against gametocytes from all species of parasite. It impairs the parasite mitochondria interfering with the ubiquinone function in the respiratory chain and also causes high production of intracellular reactive species which increases oxidative stress (Schlesinger et al., 1988; Tekwani & Walker 2006). Carboxyprimaquine has been identified as the major circulating active metabolite after oral administration of primaquine (Mihaly et al., 1984). Monoamine oxidase (MAO) is thought to be responsible for the formation of carboxyprimaquine (Jin, et al., 2014), whereas Cytochrome P450 (CYP) enzymes, especially CYP2D6 produce several ring-hydroxylated primaquine metabolites (Pybus et al., 2012). No previous studies have investigated the effects of primaquine on spermatozoa. There could be many reasons for the increase in % HA seen after incubation of spermatozoa with primaquine. Alterations in the cAMP pathway to induce an increase in the second messenger could influence initiation of HA. Also an increase in calcium conductance initiated by Primaquine or a release of intracellular calcium stores would induce HA of spermatozoa.

#### 3.4.2 40%-80% spermatozoa interface

In this study, 10 compounds were screened on donor samples, using spermatozoa from the 40%-80% interface and 80% cell fraction to determine effects on poor quality (a surrogate for patient samples) (Tardif et al., 2014) and good quality spermatozoa respectively.

Previous studies have indicated that spermatozoa from the 40% cell fraction have a similar profile - in terms of motility, morphology and DNA fragmentation index - to

infertile male spermatozoa (O'Connell et al., 2003; Glenn et al., 2007). Tardif et al., (2014) screened 46 phosphodiesterase inhibitor compounds and used 40% cell fraction as a surrogate for patient samples. Results from this study indicated that samples with initial low motility or progressive motility (40% cell fraction) showed the most significant increase after treatment with compounds whereas samples with high motility (80% cell fraction) showed significant increase in progressive motility, but only minimal effects on total motility (Tardif et al., 2014).

### 3.4.3 CASA

Using CASA to analyse cells during drug motility experiments allows for contemporaneous visual analysis of spermatozoa and generates a vast amount of data on various motility parameters. Cells can be analysed at pre-destined time points to generate an in depth picture of how the drug affects the sample over time.

Limitations of using CASA include the potential of inaccurate assessment of the cells in the sample, as estimations and percentages are calculated from a presentative number of cells in the sample rather than all cells. Given the time taken between addition of compound and initial CASA assessment of spermatozoa (5-10 seconds), it is possible that an instantaneous or transient reaction may be missed.

Only two compounds of the 10 analysed in this study which elicited an increase in  $[Ca^{2+}]_i$  were found to successfully induce spermatozoa motility parameters, specifically % HA in capacitated spermatozoa. This is a positive result with regards to potential clinical use of these compounds but further investigation must be undertaken to determine if these cells affect spermatozoa function, induce the AR or if they cause detrimental effects on cells after incubation for an extended period of time.



## **CHAPTER 4**

### **Kremer Test to Determine 10 Compounds Effects on Sperm Function**

## **Chapter 4: Kremer Test to Determine 10 Compounds Effects on Sperm Function**

### **4.1 Introduction**

Spermatozoa are deposited into the anterior vagina at coitus where they rapidly make contact with visco-elastic substances such as cervical mucus, and enter the cervix where sperm selection begins (Suarez and Pacey, 2006). Immotile and morphologically abnormal spermatozoa are unable to pass through cervical mucus and are broken down by the immune cells (Rath et al., 2008). During the progression of spermatozoa through the female reproductive tract, the selection process continues as cells must navigate the uterus, evade immune surveillance through the action of defensins: for example  $\beta$ -defensin (Yudin et al., 2005), bind to and detach from oviductal epithelium and penetrate the cumulus matrix and the ZP of the oocyte (Suarez, 2008). Therefore, sperm must employ and switch between functionally different behaviours in response to different environmental cues and stimuli. It is crucial for the spermatozoa to assume an appropriate flagellar beat pattern in response to the varying physiological conditions in order to achieve fertilisation (Stauss et al., 1995; Quill et al., 2003). Comparison of spermatozoa from the upper region of the female genital tract with spermatozoa from the ejaculate, the latter is morphologically superior with greater motility (Freundl et al., 1988), indicating that penetration of cervical mucus is dependent on these essential characteristics. Concentration of progressive spermatozoa in semen is directly correlated with the success of the spermatozoa penetration into cervical mucus (Clarke, 1997).

By adding the 10 compounds to spermatozoa *in-vitro* to increase certain motility parameters with resultant increase in the functional ability to penetrate viscous media, it was hypothesised that enhanced cell function *in-vitro* could have positive effects in ART, specifically IUI and IVF, increasing fertility rates.

#### 4.1.1 Kremer Functional Test

Testing sperm function *in-vitro* to determine its ability to penetrate cervical mucus provides a greater insight into its potential fertilising ability, as opposed to analysis of semen parameters alone (Tomlinson et al., 1999). Kremer developed a method to determine the ability of spermatozoa to penetrate cervical mucus using capillary tubes (Kremer, 1965). It was found to have many advantages, such as being a reasonable predictor of fertility of fresh human semen, if cells were able to penetrate (Fjallbrant, 1968). The Kremer test can thus determine the functional abilities of human spermatozoa and allows for a better evaluation of the prognostic value *in-vitro* (Aitken et al., 1985).

In media with low viscosity, HA spermatozoa are less progressive than activated spermatozoa, with some being completely non-progressive. However, in media of high viscosity, HA spermatozoa are more progressive than activated spermatozoa (Suarez et al., 1991; Suarez and Dai, 1992), suggesting that the induction of HA in the female reproductive tract facilitates the spermatozoa to travel in a progressive manner. The Boolean argument for mucus penetrating characteristics analysed at a frame rate of 60 Hz is as follows:  $VAP \geq 25 \mu\text{m} / \text{sec}$  AND  $STR \geq 80\%$  AND  $ALH \geq 2.5 \mu\text{m}$  AND  $ALH < 7.0 \mu\text{m}$  (Mortimer and Mortimer, 2013).

Velocity of spermatozoa progression is an important factor in determining ability to penetrate cervical mucus. A study using Kremer tests to determine spermatozoa penetration concentrations and motility characteristics in cervical mucus showed a significant correlation between concentration and specific motility parameters. Of the 30 human semen samples analysed, 83% showed normal test results when semen parameters prior to the Kremer test indicated a concentration of  $\geq 25 \times 10^6/\text{ml}$  of progressively motile cells, mean velocity of progression of  $\geq 25 \mu\text{m}/\text{sec}$  and mean ALH

of  $\geq 7.5\mu\text{ms}$ . Semen with characteristics below these limits showed abnormal penetration abilities (Mortimer et al, 1986).

Aitken et al., studied the Kremer penetration test using semen from 17 donors and oestrous bovine cervical mucus. The most important criterion that determined the success of sperm-cervical mucus interaction was ALH (Aitken et al., 1985). It was thought that the flagellar beat pattern is indirectly affected by ALH therefore this factor may be responsible for the inability to achieve adequate mucus penetration. David et al., (1981) showed that ALH is positively correlated with the size of the flagellar beating envelope. Spermatozoa showing a small ALH subsequently possesses a small amplitude flagellar beat frequency therefore rendering it incapable of generating the thrust required to penetrate cervical mucus *in-vivo* (Aitken et al., 1985).

#### 4.1.2 Spermatozoa function and infertility

Failure of spermatozoa to ascend the female reproductive tract has been detected in 45% of infertile couples (Templeton & Mortimer, 1982). Failure of spermatozoa to penetrate at the cervical level may be an important factor in this defective spermatozoa transport as shown through the use of Post coital tests (PCTs) (Aitken et al, 1985). This *in-vivo* test gives a clear indication of sperm-mucus interaction (Hull et al., 1982; Overstreet, 1986) and spermatozoa ability to ascend into the reproductive tract and fertilise an oocyte. ALH was found to be the most significant factor of spermatozoa movement that determined cervical mucus penetration success. Also, positive correlations were seen when comparing both sperm-mucus penetration and hamster oocyte penetration test outcome with ALH movement characteristics. Spermatozoa with  $\text{ALH} < 10\mu\text{m}$  were effective at penetrating mucus and hamster oocytes, but if  $\text{ALH} < 4.5\mu\text{ms}$ , cells were unable to penetrate. Some displacement of the head is required for the spermatozoa to become insinuated between the mucus chains, but if the ALH is too small there may not

be enough movement to generate sufficient propulsive force to achieve mucus penetration (Aitken et al., 1986).

The primary regulator of the spermatozoa flagellar beat pattern is  $[Ca^{2+}]_i$  signalling (Darszon et al., 2011). CatSper, the spermatozoa flagellar cation channel, is central to this process (Lishko et al., 2012). CatSper is known to be pH sensitive and weakly voltage-dependant, and in human spermatozoa it has been shown to be sensitive to progesterone (Brenker et al., 2012). Progesterone was therefore used as a positive control in Kremer penetration tests, because spermatozoa exposed to progesterone become HA, having an increased flagellar beating pattern thought to aid in penetration of cervical mucus (Alasmari et al., 2013).

#### 4.1.3 Methylcellulose as human cervical mucus substitute

The sperm-mucus interaction test has high clinical significance but it is impractical to be used as a routine clinical procedure due to difficulty in obtaining cervical mucus in sufficient quantity to perform an extensive series of tests (Eggert – Kruse et al., 1989). Replacement of cervical mucus with an artificial substitute in *in-vitro* experiments also allows standardisation of viscosity. Cervical mucus is made up of glycosylated sialo glycoprotein molecules, and the hydration of these molecules is altered during the menstrual cycle (Katz et al., 1997). This issue makes cervical mucus highly variable, as well as having instability and storage problems.

MC was used as a medium for spermatozoa penetration in place of cervical mucus. MC is cheap, is stable over long periods of time (Ray et al., 1995), and comes in various grades with varying viscosities, between 10-10,000 centipoise (cp), which covers the range of viscosities found in human cervical mucus, 2800 – 10,000 cp (Karni, 1971).

Kremer tests were used to compare MC and human cervical mucus. It was observed that MC at viscosity of 4000cp at 10mg/ml was at least as favourable to spermatozoa

penetration as human cervical mucus, allowing good discrimination between normal and subfertile samples. As the temperature was increased, the number of spermatozoa penetrating the MC increased with the optimum temperatures being around 30-37°C (Ivic et al., 2002).

#### 4.1.4 Spermatozoa sample variation

In initial Kremer penetration experiments a substantial variation in spermatozoa numbers in the Kremer tubes was seen when comparing controls from different donors, despite using a fixed cell concentration of  $10\text{--}20 \times 10^6/\text{ml}$ . The source of this variation and how to optimise the experimental technique for accurate results was also investigated.

A large variability in semen parameters exist, both between individuals and also within samples from the same individual. Clinically, to try and achieve the most accurate diagnosis when variability is seen from samples from the same individual, or the samples are suboptimal, semen samples are assessed 2-3 months apart, to allow for next cycle spermatogenesis, as it is advised that more than one semen analyses be performed before an accurate diagnosis of infertility can be made (Keel, 2006). The analysis of one semen sample from an individual is not representative of their overall fertility due to the heterogeneity within samples (MacLeod & Irvine, 1995). Because of this, researchers concluded that by increasing the number of spermatozoa donors in a study population offered more valuable information than increasing the number of semen samples per individual male (Schrader et al., 1988).

Keel et al., (2006) observed that the largest variation was seen for spermatozoa counts in both donors and patients, with 'within subjects coefficient of variation' ranging from 46% to 54% and 'between subjects coefficients of variation' ranging from 188% to well over 200%. This suggests that within subjects, the variation in the spermatozoa count

analysed from a minimum of 6 ejaculates was within a tighter range, but was still apparent, emphasising the necessity in analysing a number of samples from the one individual. The variation seen when comparing one subject with another was huge, highlighting how individual a male's semen parameters can be and the vast range of values there are observed between males. They found that individual males produced a semen sample with spermatozoa concentrations ranging from  $43 \times 10^6/\text{ml}$  to  $504 \times 10^6/\text{ml}$  and spermatozoa motility ranging from 26% to 97% (Keel, et al., 2006).

The variation in semen parameters observed in fertile males is a potential challenge for research into male fertility and something that must be considered when analysing experimental results. Alvarez et al., (2003) suggested that there are three reasons that influence the experimental results using semen samples over time; i) pre-analytical influences such as sexual abstinence period, transportation to the lab, and changes in temperature; ii) analytical random (precision) and systematic error (bias); and iii) inherent biological variation around the homeostatic setting point (within subject biological variation). Homeostatic setting points for individuals vary and this variation between individuals is called between-subject biological variation. The study concluded that conventional reference values for seminal parameters in healthy subjects have little diagnostic value because of their marked individuality and heterogeneity

To determine the source of variation in the Kremer test and whether specific cell concentration or motility characteristics affected cell penetration into viscous media, a series of experiments were designed to analyse specific parameters; i) variation in concentration of individual donor spermatozoa contained in a single Eppendorf, ii) variation in concentration of spermatozoa from a single donor split between 6 Eppendorf tubes (experimental set up for Kremer test analysis (Chapter 2.8)), iii) variation in average cell number seen in the Kremer tube at 1cm in control experiments

between different donors, and iv) whether higher cell number and/or increased progressive motility affects cell number seen at 1cm.

The aim of this study was to determine the effect that the increase in  $[Ca^{2+}]_i$  caused by the 10 hit compounds shown in the previous Flex Station screen, had on spermatozoa function using the Kremer penetration test. Spermatozoa with superior motility characteristics are able to penetrate viscous media and travel through it with greater success. It was hypothesised that compounds that improve motility characteristics would improve movement of cells through the female reproductive tracts and penetration of the ZP and oocyte membranes subsequently increasing fertilisation rates in ART. Also, the source of variation in Kremer experiments was determined by comparing various parameters.



## 4.2 Experimental Design

### 4.2.1 Determination of effect of cell concentration on percentage variation from an individual donor

An Eppendorf with 1ml of prepared spermatozoa from a single donor was analysed; 200 cells were counted using CASA giving an average concentration ( $\times 10^6/\text{ml}$ ). This was repeated 4 times and then used to calculate a percentage variation.

$$\text{Percentage Variation} = \frac{(\text{Highest concentration} - \text{Lowest concentration})}{\text{Highest concentration}} \times 100$$

### 4.2.2 Determination of variation in cell concentration ( $\times 10^6/\text{ml}$ ) (from an individual donor) between Eppendorfs

For each Kremer experiment ran, 1ml spermatozoa suspension was used with a concentration between 10 and  $20 \times 10^6/\text{ml}$  (diluted, if necessary, using capacitating media). This was split between 6 different Eppendorfs, with 100 $\mu\text{l}$  in each. Cell concentration in each Eppendorf was then determined, using CASA - 200 cells were counted 4 times to obtain an average cell concentration ( $\times 10^6/\text{ml}$ ). Percentage variation was calculated using the formula as above.

### 4.2.3 Determination of variation in cell counts at 1cm in Kremer tubes between different donors

Cells at 1cm were counted by focusing on sequential depths until all planes (and cells) in that section had been counted. This was done 4 times over the length of the tube at 1cm from the base. Of the 4 counts, an average was taken to give a cell number at 1cm for each Kremer tube (Chapter 2.8).

Percentage variation was subsequently determined using the formula:

$$\text{Percentage Variation} = \frac{(\text{Highest cell count} - \text{lowest cell count})}{\text{Highest cell count}} \times 100$$

#### 4.2.4 Determination of correlation between cell concentration ( $\times 10^6/\text{ml}$ )/progressive motility and cell number at 1cm in Kremer tubes

Data from control conditions (cells in capacitating media only) was used to compare cell concentration ( $\times 10^6/\text{ml}$ ) and percentage progressive motility of spermatozoa in the Eppendorf to the number of spermatozoa at 1cm to determine if there was a correlation.

#### 4.2.5 Kremer Variation Statistical Analysis

Scatter plots were drawn to observe the linear relationships between x and y. The Pearson Correlation Coefficient (R) was used to measure the strength and direction of the linear relationship between the x and y data sets. Statistical significance was considered  $P \leq 0.05$ . All statistical analysis was done using GraphPad Prism 5.

#### 4.2.6 Kremer Penetration Test

Kremer penetration tests were conducted as described previously (Chapter 2.8). Kremer tubes were filled with 10% MC then the top ends were sealed using plasticine to prevent evaporation. These tubes were placed into Eppendorfs containing 100 $\mu\text{l}$  of prepared 80% fraction spermatozoa from healthy volunteer donors (Chapter 2.4) at a concentration of approximately  $10 \times 10^6/\text{ml}$  cells (Chapter 4.3.1). Drug compound (or control) was added and capillary tubes subsequently gassed and heated in  $\text{CO}_2$  incubator at  $37^\circ\text{C}$  for 1 hour. Each drug was initially tested at 30 $\mu\text{M}$ . Parallel control samples included negative control (no drug added), vehicle control (DMSO; final concentration 1%), and positive control (progesterone; 3.6 $\mu\text{M}$  final concentration). Selected compounds that illustrated positive results in the previous motility screen (Chapter 3)

were also tested at 100 $\mu$ M, as this concentration induced significant increases in % HA in healthy volunteer donor spermatozoa, a motility parameter associated with enhanced mucus penetration.

After the incubation period, the capillary tubes were removed and the open ends sealed with plasticine. The tubes were marked at 1 cm and 2 cm points before being viewed on a microscope using a 20X objective microscope and CASA to count cell number.

ANOVA was subsequently used to compare control, progesterone and drug treated cells to determine statistical significance. Results were normalised to parallel untreated controls.

### 4.3 Results

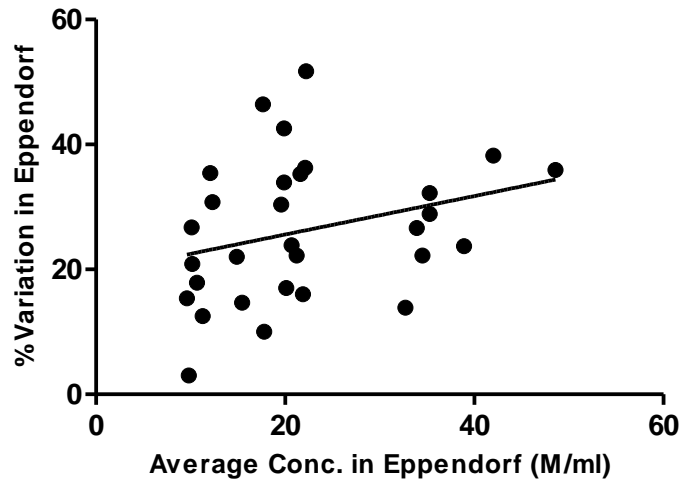
#### 4.3.1 Kremer Variation Results

Cells for the Kremer test experiments were taken from a stock of  $10 - 20 \times 10^6/\text{ml}$  cells. As the average concentration in each individual Eppendorf increases, the % variation also increases but this was not statistically significant ( $P=0.1178$ ) (Figure 4.1). No correlation was found between using cells from the same stock, distributing them into different Eppendorfs for each experiment and variation between the tubes (Figure 4.2). The consistent variation seen between Eppendorfs at all concentrations cannot be controlled for.

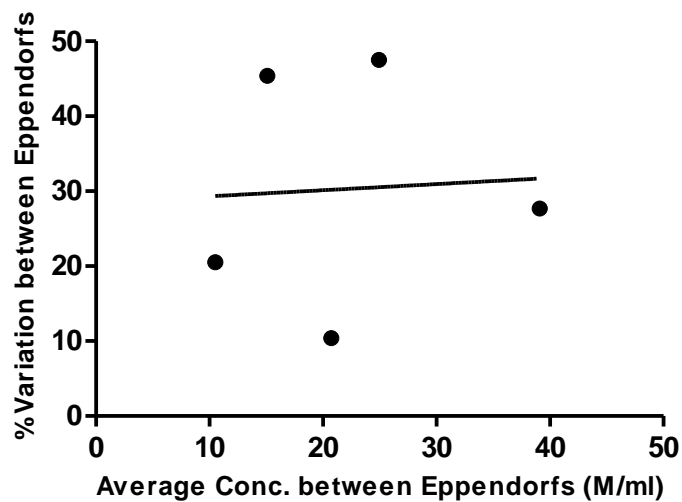
An increase in cell penetration into the Kremer tubes correlates with a reduction in variation. As the spermatozoa number counted at 1cm increases, there is a significant ( $P= 0.0053$ ) reduction in variation in the cell numbers across the length of the tube (Figure 4.3).

There is a very slight negative correlation between cell number and concentration, with the cell number at 1cm decreasing with an increase in concentration in the Eppendorf, but this was not statistically significant ( $P=0.76$ ).

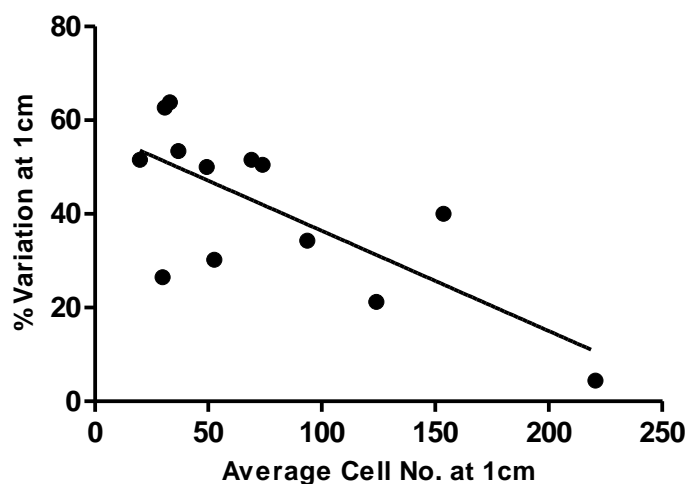
The linear relationship indicates a positive correlation between an increase in % progressive motility in the Eppendorf and an increase in cell number at 1cm, but this was not statistically significant ( $P=0.45$ ) (Figure 4.5).



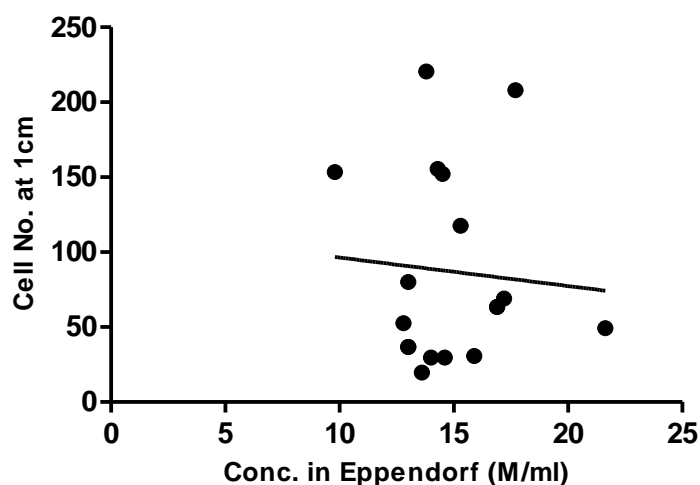
**Figure 4.1: Average cell concentration (M/ml) and % variability seen between Eppendorfs.** No significant difference ( $P=0.1178$ ) between average concentration in each Eppendorf and the % variation in each Eppendorf. Pearson Correlation Coefficient  $R=0.29$  (no linear relationship).



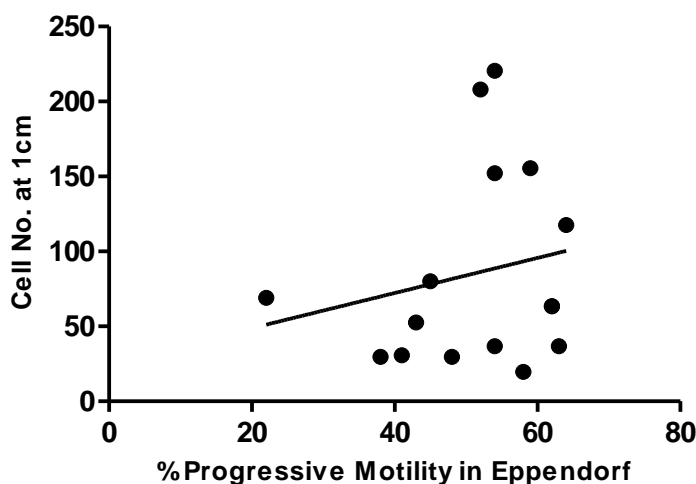
**Figure 4.2: Average concentration (M/ml) in Eppendorf and variation between Eppendorfs.** No correlation ( $R=0.056$  - no linear relationship) or statistical significance ( $P=0.93$ ) between the average concentration of cells in Eppendorfs in an experiment and the % variation between Eppendorfs.



**Figure 4.3: Average cell number at 1cm and variation at 1cm in the Kremer tube.** As the average cell number at 1cm increases the % variation at 1cm decreases significantly ( $P=0.0053$ ). Pearson Correlation Coefficient ( $R= -0.72$ ) indicates a strong downhill linear regression.



**Figure 4.4: Concentration in the Eppendorf (M/ml) compared with cell number at 1cm.** No statistical significance ( $P=0.76$ ) between cell number at 1 cm in the Kremer tube and cell concentration in the Eppendorf. Pearson Correlation Coefficient indicates no linear relationship ( $R=-0.076$ ).



**Figure 4.5: % progressive motility in the Eppendorf compared with cell number at 1cm.** No statistically significant difference ( $P=0.45$ ) between % progressive motility of cells in each Eppendorf and cell number at 1cm in the Kremer tube. Pearson Correlation Coefficient  $R= 0.202$ .

#### 4.3.2 Compounds A-J 30 $\mu$ M

When collectively analysing how drug A treated donor cells compared with control (Figure 4.6), the average normalised cell count of 4 donors indicated no significant difference when comparing drug treated with control and DMSO. Only one donor (D272) showed a significant increase in cell number when compared with control at 1cm ( $P<0.01$ ) and 2cm ( $P<0.05$ ) (Table 4.1; Appendix 9.5).

The average normalised cell count of drug B treated donor spermatozoa ( $n=5$ ) indicated no significant difference between drug B and control/DMSO treated cells (Figure 4.7). Individually, D180 indicated a significant decrease in cell number at 1cm ( $P<0.05$ ) while D258 showing a significant decrease at 2cm ( $P<0.05$ ). D093 induced a significant increase in cell number compared with control at 2cm ( $P<0.01$ ) (Table 4.2; Appendix 9.5).

Of the 8 donor spermatozoa samples screened with drug C, on average there was no significant difference between drug treated and control cells (Figure 4.8). Individually,

one donor sample (D241) showed a significant increase in cell number at 1cm ( $P<0.01$ ) and 2cm ( $P<0.05$ ) (Table 4.3; Appendix 9.5). D180 showed a significant decrease in cell number at 1cm ( $P<0.01$ ) when compared to control and Donor 093 showed a significant increase in cell number 2cm ( $P<0.05$ ).

Drug D treatment group indicates a detrimental effect on spermatozoa ability to penetrate and swim through viscous media, with the average normalised cell count ( $n=3$ ) at 1cm showing a significant decrease at 1cm when compared with control and DMSO treated cells ( $P<0.01$ ) (Figure 4.9). Two donors screened showed a statistically significant decrease in cell numbers at 1cm and 2cm (D241 ( $P<0.05$ ), D180 ( $P<0.001$ )) (Table 4.4; Appendix 9.5). Donor 266 showed no significant difference in cell count at either 1cm or 2cm in the Kremer tube.

Drug E had no effect on spermatozoa functional ability, with the average normalised cell count indicating no significant difference in cell number at 1cm and 2cm when comparing drug E with control treated cells (Figure 4.10). Individually, all 3 donor samples showed no significant difference in cell number in the Kremer tube when compared with controls (Table 4.5; Appendix 9.5).

Drug F had no effect on spermatozoa ability to penetrate and swim through viscous media with the average normalised cell count indicating no significant difference when comparing drug E with control treated cells (Figure 4.11). Individually, all 3 donor samples showed no significant difference in cell number in the Kremer tube when compared with controls (Table 4.6; Appendix 9.5).

Drug G showed no significant effect on spermatozoa functional abilities after Kremer test screening on analysis of average normalised cell count of 5 donor spermatozoa samples (Figure 4.12). Only 1 sample (D266) illustrated a significant increase in cell number at 1cm ( $P<0.01$ ) (Table 4.7; Appendix 9.5).



The majority of drug H treated samples showed no statistically significant difference in cell number at 1cm or 2cm on analysis of average normalised cell count (n=9) (Figure 4.13). Only one donor sample (D266) illustrated an increase in drug H treated spermatozoa number at 1cm ( $P<0.01$ ) when compared with control (Table 4.8; Appendix 9.5).

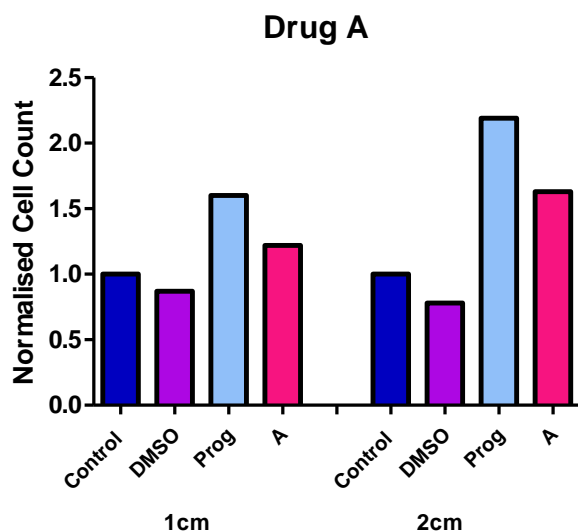
Drug I has no effect on spermatozoa ability to penetrate viscous media, with the average normalised cell count (n=5) of drug I treated cells showing no significant difference between control treated spermatozoa (Figure 4.14). One donor (D272) showed a significantly reduced cell number at 1cm ( $P<0.01$ ) and 2cm ( $P<0.05$ ) (Table 4.9; Appendix 9.5).

Drug J treated cells showed no significant difference in cell number at 1cm or 2cm when compared with control cells (Figure 4.15). One donor (D218) showed a significant decrease in cell number at 1cm ( $P<0.05$ ) when compared with control and another (D258) showed a significant decrease at 2cm ( $P<0.05$ ) (Table 4.10; Appendix 9.5).

#### 4.3.3 Compounds C+F 100 $\mu$ M

Although no increase in viscous media penetration was seen in the Kremer test experiment results after exposure to 10 compounds at 30 $\mu$ M, following analysis of motility data (Chapter 3) it was hypothesised that by increasing the concentration of specific compounds – C and F - to 100 $\mu$ M this might have a positive effect. Drug C showed only a trend towards an increase in % HA cells in the 80% cell fraction at 30 $\mu$ M, but showed a significant increase in % HA, VCL and ALH when screened at 100 $\mu$ M. Drug F also only indicated a trend towards an increase in % HA cells at 30 $\mu$ M in the 80% fraction but indicated a significant increase in % HA and ALH after exposure at 100 $\mu$ M. It was thought that these motility characteristics would contribute

to aiding sperm in viscous media penetration at 100 $\mu$ M concentration. But on analysis of Kremer test results using compounds C and F at 100 $\mu$ M (Table 4.11; Appendix 9.3.2), there was no increase in spermatozoa penetration into viscous media when compared with controls. There was either a significant decrease, or no significant difference.

4.3.4 Compounds A-J 30 $\mu$ M Results FiguresDrug A

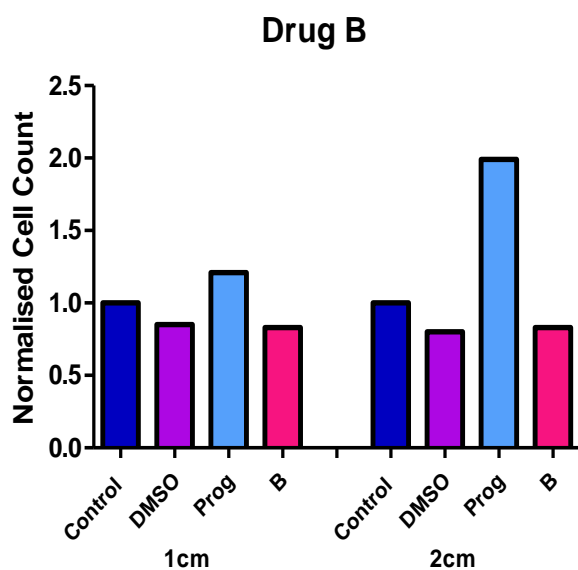
**Figure 4.6: Kremer screen of drug A 30 $\mu$ M.** No. of donors = 4. Average normalised cell number is compared using ANOVA of control, DMSO, drug A and progesterone (prog) treated cells at 1cm and 2cm. **1cm:** No significant difference. **2cm:** No significant difference.

	Drug A						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D218			✓				✓
D272	✓				✓		
D093			✓				✓
D258			✓				✓

	Progesterone						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D218			✓				✓
D272	✓				✓		
D093	✓				✓		
D258			✓		✓		

**Table 4.1: Drug A 30 $\mu$ M Kremer Test Donor Summary.** Drug A (30 $\mu$ M) and progesterone (positive control) treated cells of 4 individual donor samples; summary of results after ANOVA comparing control, progesterone and drug treated samples to determine statistical significance. >; significant increase, <; significant decrease.

## Drug B

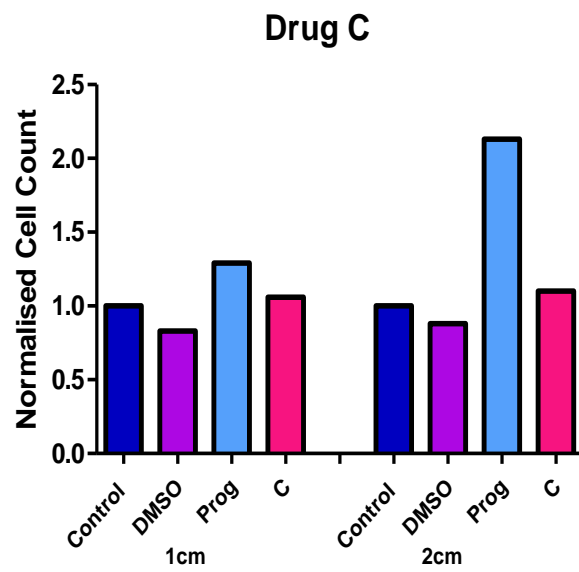


**Figure 4.7: Kremer screen of drug B 30 $\mu$ M.** No. of donors = 5. Average normalised cell number is compared using ANOVA of control, DMSO, drug B and progesterone (prog) treated cells at 1cm and 2cm. **1cm:** No significant difference. **2cm:** No significant difference.

	Drug B						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D218			✓				✓
D180		✓					✓
D266			✓				✓
D258			✓			✓	
D093			✓		✓		

	Progesterone						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D218			✓				✓
D180	✓				✓		
D266			✓				✓
D258			✓		✓		
D093	✓				✓		

**Table 4.2: Drug B 30 $\mu$ M Kremer Test Donor Summary.** Drug B (30 $\mu$ M) and progesterone (positive control) treated cells of 5 individual donor samples; summary of results after ANOVA comparing control, progesterone and drug treated samples to determine statistical significance. >; significant increase, <; significant decrease.

Drug C

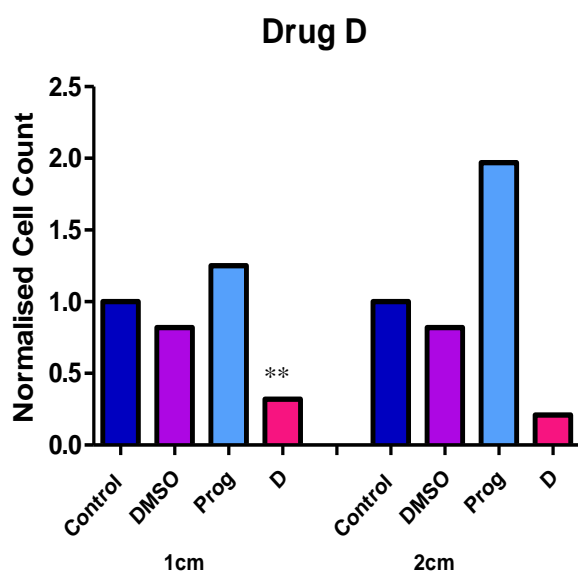
**Figure 4.8: Kremer screen of drug C 30 $\mu$ M.** No. of donors = 8. Average normalised cell number is compared using ANOVA of control, DMSO, drug C and progesterone (prog) treated cells at 1cm and 2cm. **1cm:** No significant difference. **2cm:** No significant difference.

	Drug C						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D241	✓				✓		
D180		✓					✓
D266			✓				✓
D093			✓		✓		
D266			✓				✓
D123			✓				✓
D230			✓				✓
D218			✓				✓

	Progesterone						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D241			✓				✓
D180	✓				✓		
D266			✓				✓
D093	✓				✓		
D266			✓				✓
D123			✓				✓
D230	✓				✓		
D218			✓				✓

**Table 4.3: Drug C 30 $\mu$ M Kremer Test Donor Summary.** Drug C (30 $\mu$ M) and progesterone (positive control) treated cells of 8 individual donor samples; summary of results after ANOVA comparing control, progesterone and drug treated samples to determine statistical significance. >; significant increase, <; significant decrease.

## Drug D



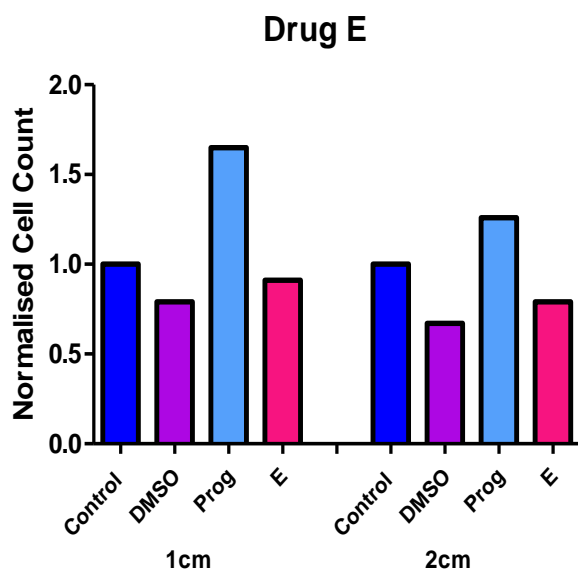
**Figure 4.9: Kremer screen of drug D 30µM.** No. of donors = 3. Average normalised cell number is compared using ANOVA of control, DMSO, drug D and progesterone (prog) treated cells at 1cm and 2cm. **1cm:** Control + drug D:  $P < 0.01$  (\*\*). DMSO + Prog:  $P < 0.05$  (\*). DMSO + drug D:  $P < 0.01$  (\*\*). Prog + drug D:  $P = 0.0002$  (\*\*\*). **2cm:** Prog + drug D:  $P = 0.0086$  (\*\*).

	Drug D						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D241		✓				✓	
D180		✓				✓	
D266			✓				✓

	Progesterone						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D241			✓				✓
D180	✓				✓		
D266			✓				✓

**Table 4.4: Drug D 30µM Kremer Test Donor Summary.** Drug D (30µM) and progesterone (positive control) treated cells of 3 individual donor samples; summary of results after ANOVA comparing control, progesterone and drug treated samples to determine statistical significance. >; significant increase, <; significant decrease.

## Drug E



**Figure 4.10: Kremer screen of drug E 30µM.** No. of donors = 3. Average normalised cell number is compared using ANOVA of control, DMSO, drug E and progesterone (prog) treated cells at 1cm and 2cm. **1cm:** No significant difference. **2cm:** No significant difference.

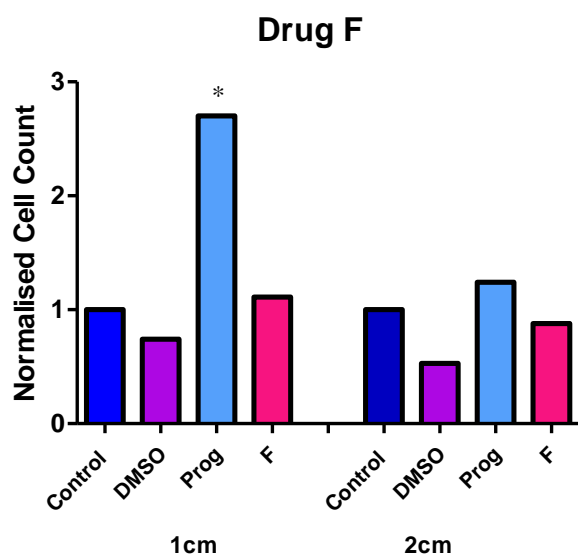
	Drug E						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D241			✓				✓
D272			✓				✓
D266			✓				✓

	Progesterone						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D241			✓				✓
D272	✓						✓
D266			✓				✓

**Table 4.5: Drug E 30µM Kremer Test Donor Summary.** Drug E (30µM) and progesterone (positive control) treated cells of 3 individual donor samples; summary of results after ANOVA comparing control, progesterone and drug treated samples to determine statistical significance. >; significant increase, <; significant decrease.



# Drug F



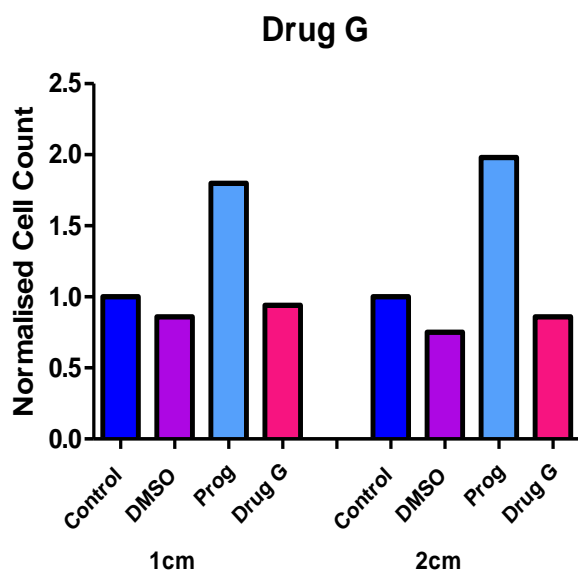
**Figure 4.11: Kremer screen of drug F 30 $\mu$ M.** No. of donors = 3. Average normalised cell number is compared using ANOVA of control, DMSO, drug F and progesterone (prog) treated cells at 1cm and 2cm. **1cm:** Control + Prog: P=0.0122 (\*). DMSO + Prog: P=0.0122 (\*). Prog + drug F: P=0.0122 (\*). **2cm:** DMSO + Prog: P<0.05 (\*).

	Drug F						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D169			✓				✓
D272			✓				✓
D266			✓				✓

	Progesterone						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D169	✓						✓
D272	✓						✓
D266			✓				✓

**Table 4.6: Drug F 30 $\mu$ M Kremer Test Donor Summary.** Drug F (30 $\mu$ M) and progesterone (positive control) treated cells of 3 individual donor samples; summary of results after ANOVA comparing control, progesterone and drug treated samples to determine statistical significance. >; significant increase, <; significant decrease.

## Drug G

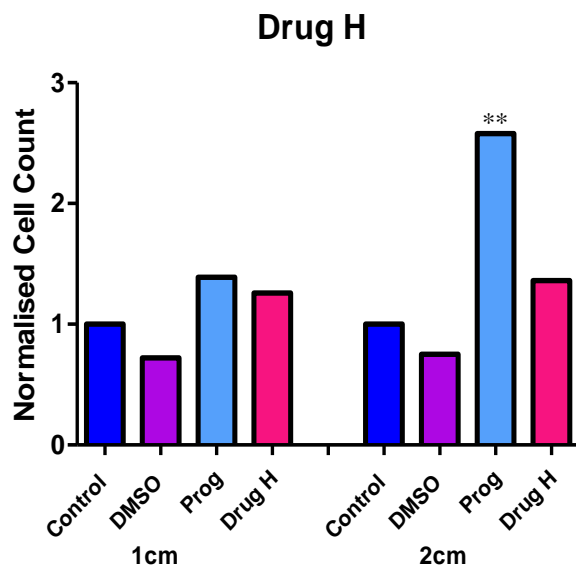


**Figure 4.12: Kremer screen of drug G 30µM.** No. of donors = 5. Average normalised cell number is compared using ANOVA of control, DMSO, drug G and progesterone (prog) treated cells at 1cm and 2cm. **1cm:** No significant difference. **2cm:** No significant difference.

	Drug G						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D169			✓				✓
D266	✓						✓
D272			✓				✓
D266			✓				✓
D272			✓				✓

	Progesterone						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D169	✓						✓
D266			✓				✓
D272			✓		✓		
D266			✓				✓
D272			✓		✓		

**Table 4.7: Drug G 30µM Kremer Test Donor Summary.** Drug G (30µM) and progesterone (positive control) treated cells of 5 individual donor samples; summary of results after ANOVA comparing control, progesterone and drug treated samples to determine statistical significance. >; significant increase, <; significant decrease.

Drug H

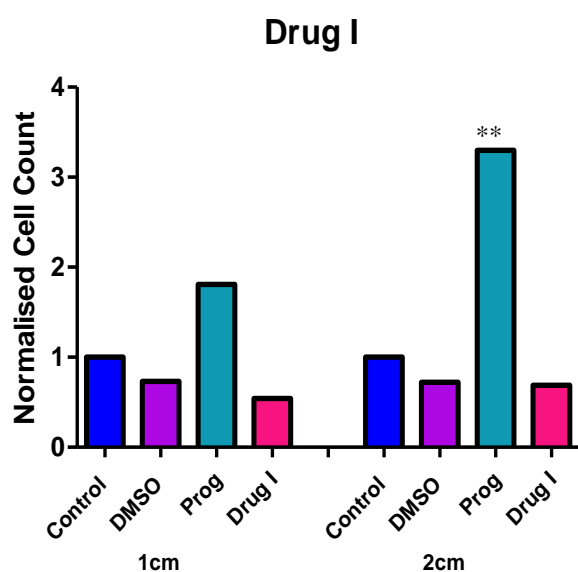
**Figure 4.13: Kremer screen of drug H 30µM.** No. of donors = 9. Average normalised cell number is compared using ANOVA of control, DMSO, drug H and progesterone (prog) treated cells at 1cm and 2cm. **1cm:** No significant difference. **2cm:** Control + Prog: P=0.0013 (\*\*). DMSO + Prog: P=0.0013 (\*\*).

	Drug H						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D169			✓				✓
D266	✓						✓
D272			✓				✓
D218			✓				✓
D272			✓				✓
D266			✓				✓
D123			✓				✓
D230			✓				✓
D218			✓				✓

	Progesterone						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D169	✓						✓
D266			✓				✓
D272	✓						✓
D218			✓		✓		
D272			✓		✓		
D266			✓				✓
D123			✓				✓
D230	✓				✓		
D218			✓				✓

**Table 4.8: Drug H 30 $\mu$ M Kremer Test Donor Summary.** Drug H (30 $\mu$ M) and progesterone (positive control) treated cells of 9 individual donor samples; summary of results after ANOVA comparing control, progesterone and drug treated samples to determine statistical significance. >; significant increase, <; significant decrease.

## Drug I



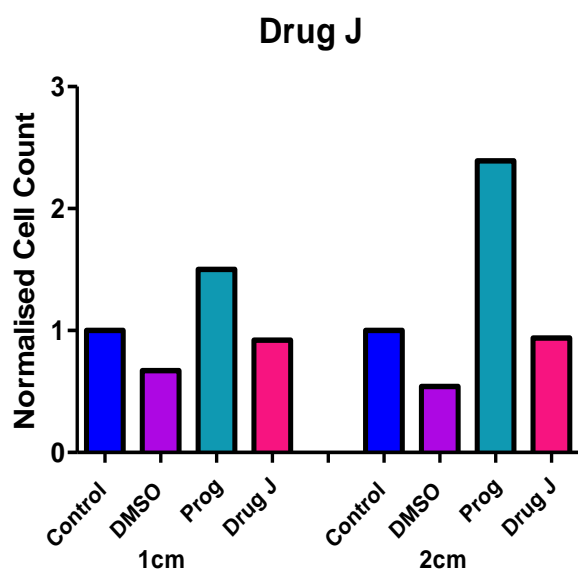
**Figure 4.14: Kremer screen of drug I 30 $\mu$ M.** No. of donors = 5. Average normalised cell number is compared using ANOVA of control, DMSO, drug I and progesterone (prog) treated cells at 1cm and 2cm. **1cm:** Prog + drug I:  $P < 0.01$  (\*\*). **2cm:** Control + Prog:  $P < 0.01$  (\*\*). DMSO + Prog:  $P = 0.0002$  (\*\*\*). Prog + drug I:  $P = 0.0002$  (\*\*\*).

	Drug I						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D266			✓				✓
D272		✓				✓	
D272			✓				✓
D218			✓				✓
D272			✓				✓

	Progesterone						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D266			✓		✓		
D272	✓				✓		
D272	✓						✓
D218			✓		✓		
D272			✓		✓		

**Table 4.9: Drug I 30 $\mu$ M Kremer Test Donor Summary.** Drug I (30 $\mu$ M) and progesterone (positive control) treated cells of 5 individual donor samples; summary of results after ANOVA comparing control, progesterone and drug treated samples to determine statistical significance. >; significant increase, <; significant decrease.

## Drug J



**Figure 4.15: Kremer screen of drug J 30µM.** No. of donors = 5. Average normalised cell number is compared using ANOVA of control, DMSO, drug J and progesterone (prog) treated cells at 1cm and 2cm. **1cm:** DMSO + Prog: P=0.0369 (\*). **2cm:** Control + Prog: P<0.05 (\*). DMSO + Prog: P=0.0077 (\*\*). Prog + drug J: P<0.05 (\*).

	Drug J						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D218		✓					✓
D272			✓				✓
D272			✓				✓
D218			✓				✓
D258			✓			✓	

	Progesterone						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D218			✓				✓
D272	✓				✓		
D272	✓						✓
D218			✓		✓		
D258			✓		✓		

**Table 4.10: Drug J 30µM Kremer Test Donor Summary.** Drug J (30µM) and progesterone (positive control) treated cells of 5 individual donor samples; summary of results after ANOVA comparing control, progesterone and drug treated samples to determine statistical significance. >; significant increase, <; significant decrease.

Compounds C + F screened at 100 $\mu$ M

	Drug C						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D202		✓				✓	
D256		✓				✓	
D267			✓				✓

	Drug F						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D202			✓				✓
D267		✓					✓
D256		✓				✓	

	Progesterone						
	1cm				2cm		
Donor	>	<	No Significant Difference		>	<	No Significant Difference
D202			✓		✓		
D256			✓				✓
D267			✓				✓

**Table 4.11: Drug C and F 100 $\mu$ M Kremer Test Donor Summary.** Drug C (100 $\mu$ M), F (100 $\mu$ M) and progesterone (positive control) treated cells of 3 individual donor samples; summary of results after ANOVA comparing control, progesterone and drug treated samples to determine statistical significance.

#### 4.4 Discussion

Donor spermatozoa samples were exposed to 10 different compounds known to induce an increase in  $[Ca^{2+}]_i$ , and Kremer function tests were undertaken to determine if any of the compounds enhanced spermatozoa functional ability to penetrate and swim through a cervical mucus substitute. Also, the source of variation in the Kremer test, how to optimise the experimental procedure to get reliable and accurate results and understand where potential error may lie was determined. The results indicate that no compound was shown to increase the ability of spermatozoa to penetrate MC, and one compound, Drug D, indicated evidence of inhibition of spermatozoa function.

##### 4.4.1 Variation in the Kremer penetration test

Although not statistically significant, it was observed that by reducing the cell concentration to  $10 \times 10^6$ /ml in the Eppendorf there was less variation when analysing different snapshots of the same sample (Figure 4.1). At this concentration, the cell sample is more homogeneous, with the cell concentration being more uniform throughout the Eppendorf. Having a constant concentration of cells in the Eppendorf will increase reliability and accuracy of cell penetration into viscous media, potentially ensuring, particularly in control experiments, less variation in cell numbers penetrating the viscous media and swimming through the Kremer tube.

As the cell number at 1cm in the Kremer tube increases, there is a significant reduction in variation between the 4 cell counts across the length of the tube ( $P=0.0053$ ). When cells move through the Kremer tube, they stick to the sides, but if there are more cells they will be forced to disperse evenly. Spermatozoa motility is influenced by surfaces, which, according to andrologists, is evident by the accumulation of cells on the surface of microscope slides and coverslips (Denissenko et al., 2012). Denissenko et al., (2012) characterised spermatozoa swimming as being ‘almost against rather than simply near



walls'. It was indicated that spermatozoa swam with their head against the wall in both pure medium and higher viscosity MC. Hydrodynamic interaction and directional self-propulsion are thought to be the most likely origin of the effect of spermatozoa swimming near to a surface (Maude, 1963; Elgeti et al., 2010). The attraction is caused by the hydrodynamic interactions between the tail and the wall, in combination with the forward thrust of the flagellar beat (Elgeti et al., 2010). Winet et al., (1984) passed spermatozoa through a 'rectangular' capillary tube (50mm long and 310 x 4000µm in lumen dimensions) and observed that a dominating characteristic with regards to spermatozoa distribution in the tube was their tendency to accumulate near to the walls. They concluded that, in tubes with a width less than 200µm an inverse relationship between calibre and frequency of spermatozoa in the centre of the tube can be expected.

It was observed that if the percentage progressive cells are greater in the Eppendorf, there is a higher number of cells penetrating into viscous media and the Kremer tube – although this correlation was not statistically significant. It has been indicated previously that the percentage of progressively motile spermatozoa in a sample directly correlates with the success of cell penetration into cervical mucus (Mortimer et al, 1986; Clarke, 1997). It can be assumed that if any of the drugs increase the progressive motility of spermatozoa, it will increase the cell number penetrating MC showing an increase in cell number in the Kremer tubes. By enhancing spermatozoa motility and optimising the experimental procedure to allow for the increase in the penetration rate into Kremer tubes and increase in cell number at 1cm (dispersing the cells more evenly across the length of the tube), this would ultimately reduce variation in results.

With regards to preferred concentration in the Eppendorf during experimental procedure,  $10 \times 10^6$ /ml cells would provide the optimum results. This concentration shows a reduced variation in the samples taken from the Eppendorf, indicating a more homogenous sample at lower concentrations. As previous evidence supported, an

increase in % progressive motility also resulted in an increase in cell number at 1cm in the Kremer tube, and this increase in cell number correlated with a significant decrease in variation at 1cm.

#### 4.4.2 Variation in response to progesterone

An apparent source of variation seen in this experiment was donor spermatozoa response to the positive control progesterone treatment. All donor samples showed an increase in cell number in the Kremer tube after progesterone treatment, but in some cases this was not statistically significant, whereas other donors indicated a highly significant response to progesterone treatment. Donors that indicated no significant difference between control and progesterone would have to conclude that the drug was not having any negative or positive effects if no significant difference was seen between control and progesterone treated cells.

Spermatozoa stay in the female reproductive tract for a period of time to undergo several  $\text{Ca}^{2+}$ -dependent physiological changes, referred to as capacitation, enabling them to fertilise an oocyte (Yanagimachi, 1994). In an individual spermatozoa sample, only approximately 2%-12% of cells become capacitated and are able to undergo the processes necessary for fertilisation such as; chemotaxis (Cohen-Dayag et al., 1995), HA and the AR (Yanagimachi, 1989). The capacitation processes are all stimulated by progesterone (Uhler et al., 1992; Villanueva-Diaz et al., 1995). Progesterone, the best characterised agonist of human sperm, is synthesised and released by mammalian cumulus (Osman et al., 1989; Yamashita et al., 2003). Progesterone acts on progesterone receptors on the cell membrane, but only a percentage of spermatozoa, 10% - 50%, express these receptors (Blackmore & Lattanzio, 1991; Tesarik et al., 1992).

Progesterone causes elevations in  $[Ca^{2+}]_i$  (Blackmore et al., 1990).  $[Ca^{2+}]_i$  signalling is vital to spermatozoa function (Jimenez-Gonzalez et al., 2006), and the stimulation of human spermatozoa with progesterone activates plasma membrane receptors (Blackmore et al., 1991) resulting in the rapid increase in  $[Ca^{2+}]_i$  in nearly all cells (Harper et al., 2003). Spermatozoa express a range of plasma membrane  $Ca^{2+}$  permeable channels and are thought to possess  $Ca^{2+}$  stores (Darszon et al., 1999; Ren et al., 2001).

Spermatozoa in a sample have been shown to respond variably to progesterone. Because a semen sample is heterogeneous, the measurement of calcium concentration in the whole sample is not representative as only a subpopulation of the spermatozoa in the sample are able to increase  $[Ca^{2+}]_i$  (Giojalas, 1998). Giojalas, (1998) illustrated that the percentage of cells that showed an increase in  $[Ca^{2+}]_i$  in response to progesterone was greatest in the subpopulation showing a high percentage motility. The results also indicated that the size of the progesterone-responsive subpopulation varies, for example, although there is a correlation between the percentage of viable spermatozoa and  $[Ca^{2+}]_i$  response to progesterone, not all viable cells respond to the hormone stimulus.

There is a correlation between the responsiveness of human spermatozoa to progesterone and the success rates of fertilisation *in-vitro* (Krausz et al., 1996).

Spermatozoa response to progesterone has been considered a good marker as semen samples from infertile patients show a very low or no progesterone response (Oehninger et al., 1994; Kotwicka & Warchal, 2001). Giojalas et al., (2004) found, using FCM that samples from subfertile males indicated a low response to progesterone. In IUI semen samples an average of 50% of spermatozoa responded to progesterone, and this decreased in IVF and ICSI samples.

#### 4.4.3 Advantages of using sperm-mucus function tests

Although used regularly in a clinical setting, the conventional methods of semen analysis are insufficient to accurately predict spermatozoa fertilisation capacity or to determine the success of an ART cycle, so spermatozoa function tests have been developed, such as the sperm-mucus penetration test (Varghese et al., 2005), to predict more accurately spermatozoa dysfunction.

The analysis of spermatozoa interaction with cervical mucus is very important as it is the first barrier in the transport of spermatozoa into the female reproductive tract *in-vivo*. It has been suggested that this natural cervical mucus barrier enables the selection of poor quality spermatozoa allowing only the most fertile cells to pass through into the reproductive tract (Eggert-Kruse et al., 1995). There have been many positive results reported from studies undertaking *in-vitro* sperm-cervical mucus penetration tests, showing positive relationships between mucus penetration and progressively motile spermatozoa with regards to IVF outcome (Berberoglul et al., 1993). Specific male factors which influence the outcome of the CM penetration test include; movement characteristics of spermatozoa (velocity, linearity and ALH), the presence of anti-sperm antibodies in the mucus, initial spermatozoa concentration and spermatozoa morphology (Boyers, 1989; Hellstrom, 1989).

Using the Kremer test with this MC viscosity is a very good indicator of potential cell function ability *in-vivo*, however there are limitations, such as in IFV and IUI the penetration of cervical mucus is bypassed in both treatments. The cells do not have to pass through media of high viscosity. But, it still stands that viscous media penetration is a good indicator of cell function and similar movement parameters required to penetrate cervical mucus substitute, such as HA and ALH, are also required to penetrate cumulus cells, ZP and oocyte membranes. This function test would indicate fertilisation

potential of cells *in-vitro*, therefore if the drugs were able to induce penetration into Kremer tubes, the increase in functional ability would aid in penetration of cumulus cells and oocyte membranes in IUI and IVF and so increase success rates of cycles.

#### 4.4.4 Spermatozoa sample variation

As indicated previously (Chapter 4), there is a large variation in cell number found in the Kremer tube even when controls are set for a specific concentration in the Eppendorf at the beginning of the experiment. A study using an altered version of the Kremer test, the QMPT, found that the concentration of spermatozoa collected from 21 donors after QMPT ranged from 3.2 to  $89 \times 10^6/\text{ml}$ . QMPT result was correlated most strongly with the concentration of progressive (VAP  $>30 \mu\text{m/s}$ , STR  $>90\%$ ) spermatozoa in the donor semen, determined by CASA (Clarke, 1997). Because individual donor samples show variation in their spermatozoa parameters; for example percentage motility, progressive motility, rapid cells and ALH – this will affect cell number penetrating the cervical mucus and so individual donor response was also analysed separately to determine how the compounds affected individual spermatozoa samples functional abilities.

#### 4.4.5 Reduced response in the Kremer test

Drug D indicated that it was having a detrimental effect on donor spermatozoa function with a significant reduction in spermatozoa number at 1cm ( $P<0.01$ ) and the majority of donor samples (2 out of 3) illustrated a significant reduction in spermatozoa number at 1cm and 2cm when compared with control. Drug D, Trifluoperazine, is an anti-anxiety drug used to treat schizophrenia. It has varying degrees of dopaminergic activity, known to block postsynaptic mesolimbic dopamine receptors D1 and D2 in the brain.

Dopamine, a member of the catecholamine family, is an important neurotransmitter in the mammalian central nervous system (Missale et al., 1998). There are two types of

dopamine receptors, type 1 (D1 like receptors – DRD1 and DRD4) and type 2 (D2 like receptors - DRD2, DRD3 and DRD4), which are all 7 transmembrane GPCRs (Missale et al., 1998). Stimulation of D1-like receptors activates AC, increasing cAMP accumulation activating PKA (Himmler et al., 1993; Das, et al., 1997). But, stimulation of D2-like receptors (which antipsychotic drugs show high affinity for), inhibits AC by coupling to the inhibitory GTP-binding regulatory protein and decreases PKA activity (Missale et al., 1998).

Catecholamines have been detected in human semen (Fait et al., 2001), and some neurotransmitter receptors have been detected on mammalian spermatozoa (Meizel et al., 2004), for example DRD2 expression in the rat (Otth et al., 2007). This could indicate a potential target for endogenous dopamine and antipsychotic drugs. Ramirez et al., (2009) used immunofluorescence to reveal DRD2 in fresh boar semen; it is detectable in the flagellar region, largely in the midpiece. However, after 2-hours incubation in capacitating media, DRD2 indicated strong immunoreactivity in the acrosomal region. The researchers concluded that dopaminergic agonists' effects on spermatozoa function occur at two levels. Firstly dopamine ( $<10\mu\text{M}$ ) increases tyrosine phosphorylation, viability and motility by activation of DRD2. Secondly, at high concentrations of dopamine (1mM) decreased tyrosine phosphorylation and motility by a mechanism involving dopamine uptake by a dopamine transporter, by cytosolic accumulation of dopamine, or by enzymatic oxidation or auto-oxidation of dopamine (Ramirez, et al., 2009).

The dopamine receptor D2 was shown to be expressed along the tail and the acrosomal region of rat, mouse, bull and human spermatozoa by fluorescent immunolocalisation. D2-like dopamine receptors share a high affinity for antipsychotic drugs and they couple to the receptors through pertussis toxin-sensitive G proteins ( $G_{i/o}$ ) (Otth et al.,

2007). The blocking of dopamine receptors by Trifluoperazine may have caused severe detrimental effects by inhibiting tyrosine phosphorylation and inducing cell death.

#### 4.4.6 Conclusions

The lack of response of spermatozoa shown in the Kremer functional test after exposure to 10 compounds known to induce an increase in  $[Ca^{2+}]_i$  indicates that they do not enhance spermatozoa function by enabling penetration and movement through viscous media. One compound even illustrated a detrimental effect on cell function. It was hypothesised that if a compound could enhance spermatozoa motility and function, this could be used clinically to increase fertilisation rates of IVF and IUI therefore increase the number of couples achieving pregnancy and live births through ART.

## **CHAPTER 5**

# **Flow Cytometry Analysis of Acrosome Induction and Cell Death**



## **Chapter 5: Flow Cytometry Analysis of Acrosome Reaction and Cell Death**

### **5.1 Introduction**

Spermatozoa are stimulated to undergo the AR *in-vivo* when in the proximity of a mature oocyte (Darszon et al., 2011). The AR is a specific secretory event that occurs in spermatozoa undergoing capacitation, and is characterised by significant changes in the topology of the plasma membrane and protein distribution. It is required for sperm-oocyte recognition and fusion (Yanagimachi, 1994; Inoue et al., 2011). Acrosome intact spermatozoa reach the ZP and its components (ZP1 – ZP4) promote the AR by interacting with spermatozoa surface molecules. Following binding to the ZP, outer acrosomal membranes fuse with the spermatozoa plasma membrane allowing acrosomal contents ie enzymes (acrosin) to be released in an exocytotic process, enabling penetration of the ZP (Anifandis et al., 2014). *In-vivo*, the stimulation of spermatozoa by the mature oocyte to undergo the AR induces a complex signalling cascade which triggers an increase in  $[Ca^{2+}]_i$ , including an initial transient increase followed by a sustained increase (Costello et al., 2009; Darszon et al., 2011). Progesterone is a physiological inducer of the AR in mammalian spermatozoa during *in-vivo* fertilisation (Baldi et al., 2011; Jin et al., 2011) and acts by inducing a rapid influx of  $[Ca^{2+}]_i$  (Foresta et al., 1993; Publicover et al., 2008). CatSper channels are activated by progesterone, stimulating AR in human spermatozoa (Publicover et al., 2008; Strunker et al., 2011; Tamburrino et al., 2014).

Both internal and external calcium are vital for the AR (Yanagimachi, 1994; Rossato et al., 2001). The entry of calcium into spermatozoa is thought to regulate biological processes that are vital for the AR (Rossato et al., 2001), including tyrosine phosphorylation and the activation of multiple kinase pathways (Sagare-Patil et al., 2012).

However, if spermatozoa AR prematurely, this renders them unable to fertilise (Zoppino et al., 2012). The AR is an essential for successful fertilisation, however it must occur at the appropriate time and in the proximity of the oocyte (Barbonetti et al., 2008). After the AR has occurred *in-vitro*, reduction in motility can be observed (Harper et al., 2008), suggesting that this may be the mechanism by which premature AR *in-vivo* (or *in-vitro*) renders spermatozoa unable to penetrate the vestments to reach the oocyte and thus unable to fertilise. It has been observed that chemically induced premature AR reduces rat spermatozoa's ability to penetrate zona intact oocytes. This reduction was not observed when the zona was removed, indicating a negative linear relationship between the frequency of AR spermatozoa and the capabilities of sperm-oocyte binding and penetration in zona-intact oocytes (Hsu et al., 1999). If the AR were to occur prematurely in IUI or IVF cycles, the cells would be unable to penetrate the ZP and fertilise the oocyte, therefore causing detrimental effects on ART outcomes. Of concern, therapeutic approaches to induce a significant increase in  $[Ca^{2+}]_i$  may also initiate AR, which would be a detrimental effect and would preclude use in clinical practice.

#### 5.1.1 Studying the Acrosome Reaction

There are several techniques that have been developed to assess the AR in spermatozoa, most being based on the binding of fluorescent acrosome specific probes in fixed cells (Cross and Meizel, 1989). The method of analysing acrosome reacted fixed cells is very time consuming and subjective – visually counting the number of fluorescent cells on the slide. Alternatively, FCM provides a more accurate and rapid method of assessing the AR in spermatozoa and allows for analysis of a very large number of cells (Hossain et al., 2011).

The use of fluorescent reagents that recognise the acrosomal content in live cells is restricted because the interior of the granule is isolated from the medium by the

acrosomal and plasma membranes. Therefore cells must be fixed and permeabilised to allow for staining. However, incorporation of acrosomal markers soy-bean trypsin inhibitor (SBTI) and anti-CD46 antibody during exocytosis has been used to detect the AR in living human spermatozoa (Harper et al., 2008). The technique uses the phenomenon where opening of fusion pores connects the extracellular medium to the acrosomal lumen at early stages of exocytosis of the acrosome. SBTI has a high affinity for the acrosomal protease acrosin, and when added to incubation medium it labels the acrosome when the fusion pores open (Harper et al., 2008).

Another marker used to assess AR in fixed (Mendoza et al., 1992) and unfixed (Farlin et al., 1992) cells is PSA, a dimeric lectin from the pea plant that binds to alpha-mannose and alpha-galactose moieties of the acrosomal matrix (Cross et al., 1986). This lectin - fused with fluorescein isothiocyanate (PSA-FITC) recognises many of the highly glycosylated proteins present in the acrosome and is known to stain the acrosomal region of fixed and permeabilised unreacted sperm. PSA and SBTI have a similar mechanism of fusion kinetics, but the PSA lectin label fluoresces for a longer period of time (Zoppino et al., 2012).

Spermatozoa imaged using time-lapse imaging in a confocal microscope in the presence of PSA-FITC, show a rapid increase in fluorescence. After the initial increase, the fluorescence continues increasing at a slower speed and remains sperm-associated. PSA-FITC labelling does not decrease over time. The lectin cross links the acrosomal content, preventing the diffusion of the material, and binding the vesicles to the cell (Zoppino et al., 2012).

In fixed and permeabilised spermatozoa, PSA-FITC binds to the intact acrosome. AR spermatozoa lose the lectin binding material during exocytosis. Therefore, cells that fluoresce are the cells that have not undergone the AR. By contrast, in live cells, PSA-

FITC is incorporated during exocytosis. Determination of the AR spermatozoa is therefore achieved by counting the number of cells that become fluorescent during incubation (Zoppino et al., 2012).

A recent study by Escoffier et al., (2015) genetically modified mice to carry Acr-GFP in their spermatozoa acrosomes. They used flow cytometry to determine the status of the acrosome in subpopulations of capacitated spermatozoa with different membrane potentials. Hyperpolarised cells are almost completely made up of GFP positive (acrosome intact) spermatozoa, whereas depolarised cells were GFP negative (acrosome reacted).

#### 5.1.2 Flow Cytometry

Live cells tagged with PSA-FITC, fluoresce when undergoing the AR. Zoppino et al., used FCM to analyse the AR cell population. FCM is a technique used to measure physical and multicolour fluorescent properties of cells flowing in a moving stream. Many cells within a heterogeneous population can be analysed. A flow cytometer is made up of four main systems; fluidics, optics, electronics and software handling.

The cell suspension flows through a tubular system (fluidics). Thousands of cells per second are exposed to a laser/lasers that differentially excite uploaded markers (fluorophores) through the absorption of light energy, resulting in the emission of light energy from fluorophores (fluorescence). A given fluorophore, has a wavelength for the peak of the illumination excitation intensity and a wavelength for the peak of fluorescence emission intensity. FCM detects fluorescence labelling by multiple fluorophores associated with individual spermatozoa, so that more than one spermatozoa attribute can be assessed simultaneously. The emission of light from the cell is the fluorescence of labelled fluorophores, and gives information about cell size and complexity. The recorded emissions from the laser illumination of the cells are

gathered and directed (optics) to detectors that receive the light. These detectors then convert light intensity into voltage. The magnitude of the voltage pulse recorded for each cell is proportional to the cell properties such as fluorescence, absorbance, and light scattering, and it is then digitalised (electronics), by converting voltage signals from the detectors into digital data. The data is analysed by computer (Flow Jo software; software handling) to provide visual information about a cell sample. Data can be acquired from different subpopulations within a sample, therefore evaluating heterogeneous populations in different states of activation. As a result, the analysis becomes objective, has a high level of experimental repeatability and also has the advantage of being able to work with large sample sizes (Hossain et al., 2011).

The previously described Flex Station screen indicated that all 10 compounds induced an increase in  $[Ca^{2+}]_i$  in human spermatozoa. The implication of this increase in  $[Ca^{2+}]_i$  could potentially be induction of AR (Breitbart & Spungin, 1997), which would be a detrimental effect rendering the compounds unsuitable for use in a clinical (*in-vitro*) setting. The aim of this experiment was to assess the use of a published method (Zoppino et al., 2012) to measure the AR and cell viability in human spermatozoa using FCM, after exposure to compounds identified from HTS that induce an increase in  $[Ca^{2+}]_i$ . This approach also estimated the percentage of cells that were reacting and therefore the potency of the compounds. Cell viability after exposure to the compounds for 60 minutes was also analysed using a cell death marker (TO-PRO3). It was hypothesised that the effect of this influx of  $[Ca^{2+}]_i$  caused by the drug compounds did not represent induction of AR in human spermatozoa.

## 5.2 Experimental Design

Donor spermatozoa were prepared by DGC as described previously (Chapter 2.4). The FCM assay was made up in standard Flex Station assay buffer, as used previously in earlier experiments (Chapter 2.6). Briefly, cells were incubated with PSA-FITC and compounds A-J (30 $\mu$ M) for 60 minutes. The AR is a relatively slow process, with the percentage of AR cells increasing with time of incubation in the presence of physiological and pharmacological stimulation (Harper et al., 2008). It is thought that the reaction takes around 60 minutes to reach a plateau (Sosa et al., 2015). Calcium ionophore A23187 was used as a positive control, ethanol (used to dissolve A23187) and 1% DMSO were used as vehicle controls. TO-PRO3 stain was used as a cell death marker. FITC has excitation and emission peak wavelengths of approximately 495 nm/519 nm respectively. TO-PRO3 has excitation and emission peak wavelengths of approximately 642 nm/661 nm respectively. FCM recordings were taken at time 60 minutes (T60) using a FACs canto machine. 10,000 events (cells) were analysed at each recording. Flow Jo computer software package was used to analyse the results of the flow cytometry screen.

Inducing calcium influx into spermatozoa using a calcium transporting agent (as a positive control), such as the calcium ionophore A23187 - valuable for pharmacological stimulation of the AR in capacitated human sperm (WHO, 1999) - allows the induction and study of the AR *in-vitro* (Aitken et al., 1993). To achieve the stimulation of the AR *in-vitro*, the *in-vivo* cellular processes that occur - an influx of extracellular calcium and a rise in intracellular pH - are imitated (Fraser and Ahuja, 1988; Garbers, 1989). The calcium ionophore A23187 induces these changes in mammalian sperm (Summers et al, 1976; Talbot et al. 1976). However, the concentration of A23187 used, and length of exposure, must be carefully regulated because it can be cytotoxic at high doses (Aitken et al., 1993). Some spermatozoa samples show a varied response to progesterone

(Giojalas, 1998) therefore calcium ionophore was used as a fail-safe method of inducing the AR in a large number of spermatozoa, making it a good positive control.

#### 5.2.1 Cell size and morphology: Forward Scatter against Side Scatter plot

An individual cell population can be distinguished from another by the distribution of dots on the dot plot. This allows for the gating of particular populations of cells for further analysis. The forward-light-scatter gate was set to allow determination of the total cell population whilst excluding cell debris or aggregates. Thus when plotting a scatter plot of FSC against SSC, the distribution of cells to be analysed is based on size and morphology respectively. Larger cells are represented as higher values along the FSC axis, while cells that are more granular (more objects inside the cell to refract the laser) are represented as higher values along the SSC axis. In a spermatozoa sample the cells that are referred to as single cells are the morphologically normal cells. These are gated out and analysed separately according to their position on the FSC SSC plot. The majority of spermatozoa appear to have a low SSC because they only contain a nucleus, and therefore not a lot of organelles to refract the laser. They also appear to have a low FSC because of their small size. Remaining cells are debris from the spermatozoa fraction, and comprise morphologically abnormal cells that are not of the same shape and size as the spermatozoa.

#### 5.2.2 Forward Scatter against Single Fluorescent Dye Plot

The gated single cells of similar size (FSC) were plotted against the wavelength of TO-PRO3 fluoresced by each cell to determine the population of live and dead cells.

By looking at single colour data using a dot plot, variances in wavelength of the fluorophore (in this case TO-PRO3) expressed by each cell can be observed. As only the gated single cell population of specific size and complexity (sperm cell population)

are analysed in this dot plot, their FSC value is within a narrow range, but the TO-PRO3 fluorescence is highly variable therefore there are clear populations of live and dead cells. The dead cell population appeared to emit a higher wavelength of light from the TO-PRO3 fluorophores, presumably because the dye penetrates the cell membrane and stains the nucleus of the dead cells. The live cells emit much lower wavelengths of light because their cell membranes are still intact. Cells that are dying can be seen to be emitting a wavelength of light in between both of these values due to the varying levels of TO-PRO3 staining of the cell nucleus.

### 5.2.3 Two Fluorescent Dye Plots

A single dot plot was used to analyse multiple fluorophores (PSA-FITC; TO-PRO3). Only the gated live cells were analysed and these all emitted a TO-PRO3 wavelength within a narrow range, thus allowing determination of the percentage of acrosome and non-acrosome reacted cells in the population, based on varying wavelengths of FITC detected.

### 5.2.4 Statistics

ANOVA (analysis of variance) test was used for statistical comparison of drug, ionophore and vehicle control treated cells. Tukey test was used post ANOVA to indicate statistical comparisons between groups.



### 5.3 Results

Of the 6 donors treated with A23187, four showed a large percentage of AR cells (38.5% (Figure 6.1), 46.7% (Figure 6.2) 40.3%, and 33.7%) (Appendix 9.4). The other two donors showed a reduced response (11.6% and 10%). The number of AR cells in the A23187 treated samples was statistically significantly greater ( $P < 0.0001$ ) than DMSO and ethanol treated cells. The percentage dead cells of the 6 donors incubated with A23187 for 1 hour was variable, with the lowest being 23.4% (Figure 6.1) and the highest being 35.5%. The percentage cell death for the other donors was; 29.1%, 25.2%, 34.6%, 33.7% (Figure 6.2; Appendix 9.4). There was no significant difference in cell death when comparing A23187 with DMSO and ethanol treated cells.

DMSO did not induce the AR, with only a very small percentage of live cells fluorescing with FITC-A after 1 hour incubation in all donors (2.26%, 1% (Figure 6.3), 2.53%, 1.8% (Figure 6.4), 3.32%, 0.71%) (Appendix 9.4). Donor cell death after incubation with spermatozoa for 1 hour was; 42.9% (Figure 6.4) 29.6%, 27.9% (Figure 6.3), 24%, 25.4%, 15.9% (Appendix 9.4).

The percentage dead cells after 1 hour incubation with ethanol in all the donor experiments was; 33.5%, 20.8% (Figure 6.5), 33.6% (Figure 6.6), 26.2%, 30.9% and 17.9% (Appendix 9.4). There was no significant difference between ethanol and DMSO treated cells. The percentage of AR cells in the live cell population of all the donors screened with ethanol were; 4.42% (Figure 6.6), 2.27%, 0.3% (Figure 6.5), 4.13%, 4.75%, 1.29% (Appendix 9.4).

Drug A did not induce the AR in any donor sperm sample after 1 hour incubation with the three samples; 0.87% (Figure 6.7), 2.07% and 1.33% AR cells (Appendix 9.4), as there was no significant difference between drug A and control treated cells. Drug A induced 35.6% (Figure 6.7), 25.1%, and 18.1% (Appendix 9.4) cell death in three

separate donor samples after incubation for 1 hour, similar to vehicle controls – no significant difference.

Drug B did not induce the AR in human spermatozoa, with the AR cell population in the three donor samples screened being 1.45% (Figure 6.8), 1.32% and 1.1% (Appendix 9.4) and no significant difference between drug A treated and control cells. The percentage of dead cells in each of the donor populations after drug B; 39.1% (Figure 6.8), 27.3%, and 24.1% (Appendix 9.4), indicated no induction of cell death when compared with controls.

Drug C shows no indication of inducing the AR, with AR cell population being 0.495% (Figure 6.9), 1.42%, and 1.49% (Appendix 9.4) in each of the three donors – no significant difference from controls. The percentage of dead cells is 33.5% (Figure 6.9), 30% and 24.7% (Appendix 9.4) after 1 hour incubation with Drug C, similar to control experiments.

Drug D is having a very detrimental effect on spermatozoa, as after 1 hour of incubation with the compound 93% (Figure 6.10), 95.7% and 85.4% (Appendix 9.4) of the three donor sperm cells have died. There is a significant increase in cell death in Drug D treated cells when compared with DMSO and ethanol ( $P < 0.0001$ ). This left a very small number of live cells for analysis of the AR, but it was shown that 3.38% (Figure 6.10), 1.25% and 2.51% (Appendix 9.4) of cells were AR after 60 minutes incubation with drug D, which is similar to controls (no significant difference), indicating the drug does not induce the reaction.

Drug E does not induce the AR in the live cell population, with the AR cell populations being 1.18% (Figure 6.11), 1.2%, and 1.98% (Appendix 9.4) in each donor, similar to control treated cells (no significant difference). Drug E does not induce cell death, to

any greater extent than controls (no significant difference), with 38.1% (Figure 6.11), 29.8% and 28% (Appendix 9.4) cells were dead after 1 hour of incubation with drug E.

Drug F is having no significant effect on sperm viability, with the percentage of dead cells in each of the donor samples after 1 hour incubation being; 28.5% (Figure 6.12), 37.3% and 30.5% (see appendix 9.4), no significant difference from controls. When analysing the live cell population it was shown that only 1.71% (Figure 6.12), 3.23% and 1.84% (Appendix 9.4) of the three donors' cells were AR, no significant difference from control populations.

Drug G had a severely detrimental effect on the viability of spermatozoa, as after 60 minutes of incubation with the compound, 100% of the single cell population in all three donors were dead (Figure 6.13; Appendix 9.4). This is a significant increase when compared with control treated cells ( $P < 0.0001$ ). This cell death left no cells to be analysed for induction of the AR because after one hour incubation with drug G there were no live cells in any sample, therefore the % population of cells which had undergone the AR could not be analysed in all three donors (Figure 6.13; Appendix 9.4).

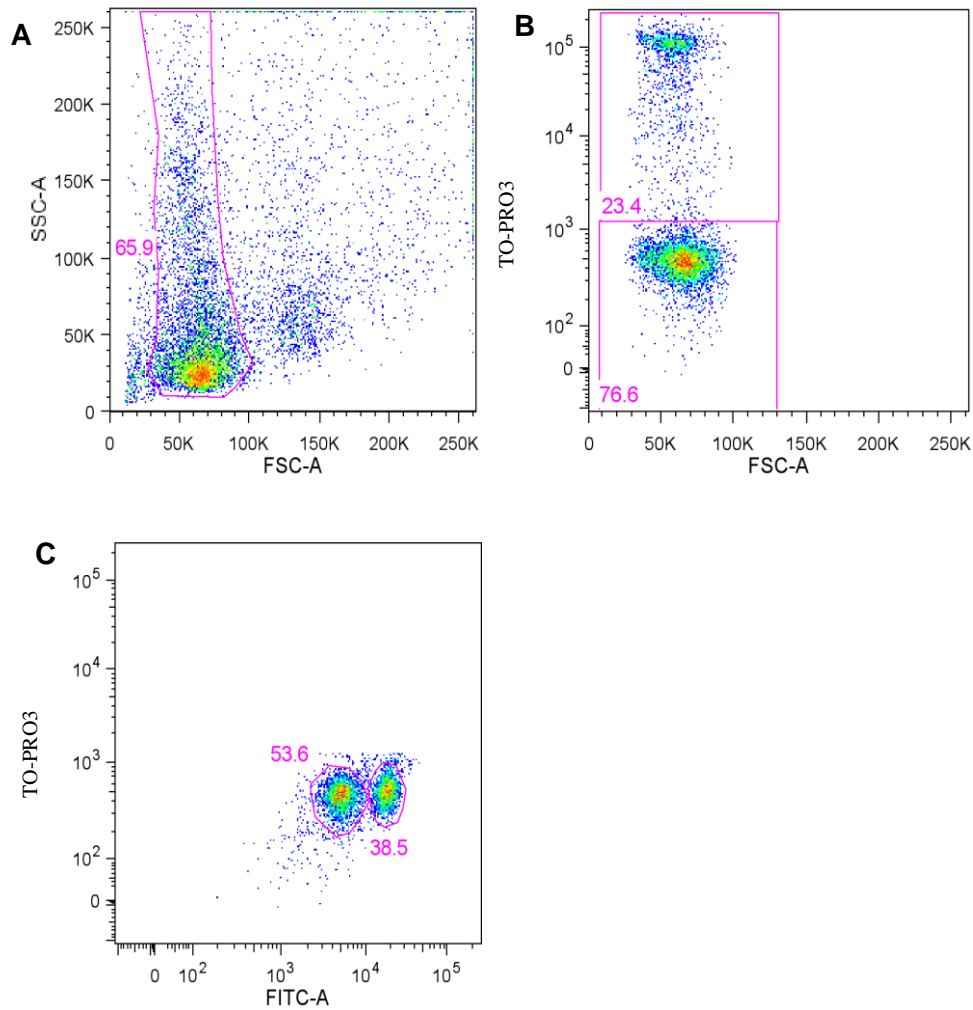
Drug H does not induce the AR, with the three donors having 1.47% (Figure 6.14), 0.924% and 0.799% acrosome reacted cells after 1 hour of incubation with drug H (Appendix 9.4), no significant difference from controls. In the single cell analysis, there are 42.1% (Figure 6.14), 25.2% and 29.9% dead cells in the three donor cell populations after 60 minutes of incubation with compound H (see appendix 9.4), which was not significantly different from control treated cells.

Drug I also does not induce the AR, with 4.16% (Figure 6.15), 1.04% and 1.38% (Appendix 9.4) of the three donor live cell populations undergoing the AR, no significant difference when compared with controls. The percentage dead cells in the

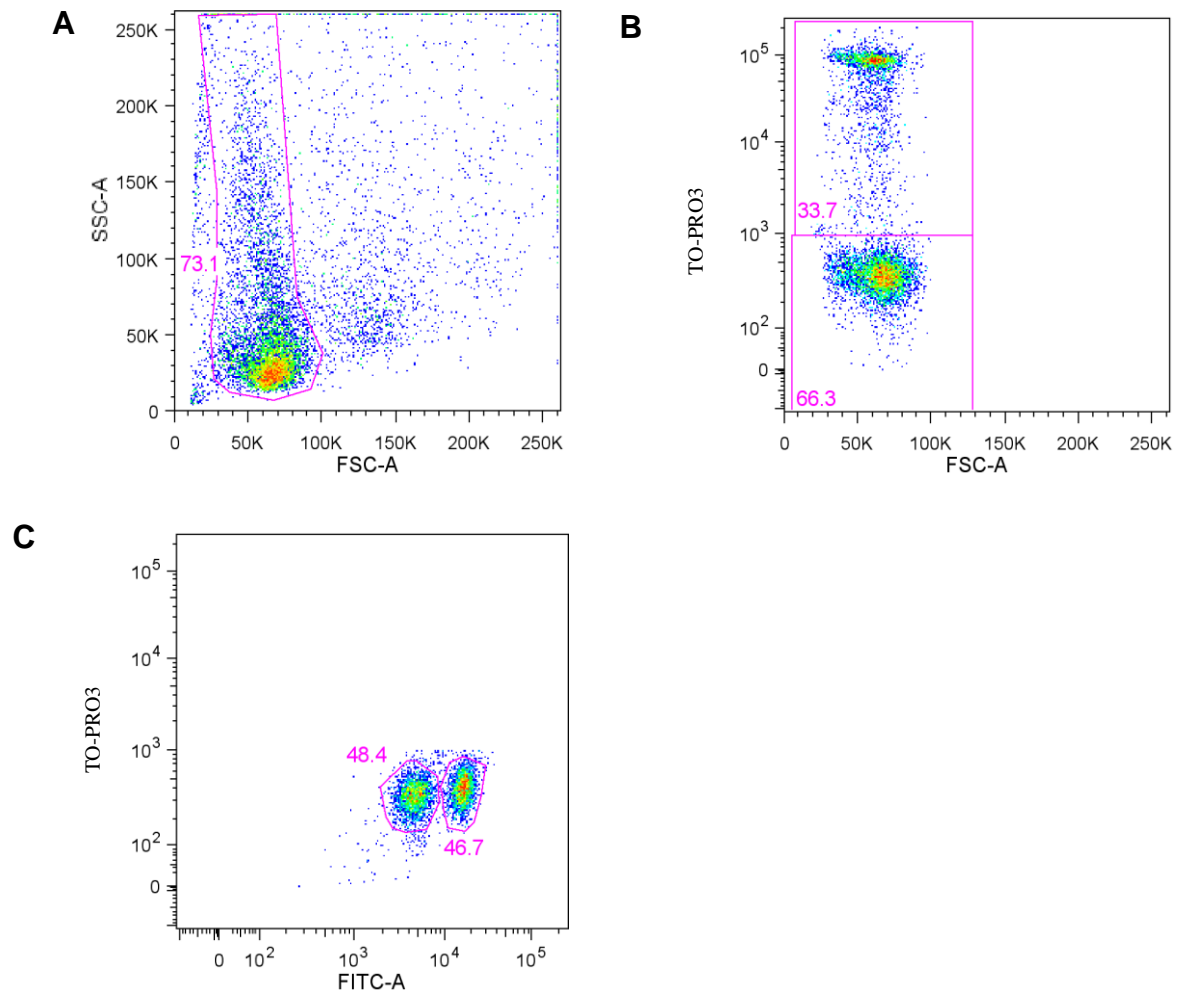
single cell populations of the three donors; 55.5% (Figure 6.15), 36.1% and 45.8% (Appendix 9.4) is significantly high compared to control experiments ( $P=0.0119$ ), suggesting the compound is having a slight detrimental effect on the spermatozoa viability.

Drug J does not induce the AR in live spermatozoa as the population of AR cells in the live cells of three donors was; 3.57% (Figure 6.16), 1.76%, and 0.67% (Appendix 9.4), no significant difference from control samples. When analysing dead cells, one donor showed a higher population, 40% (Figure 6.16), than the other two - 27.8% and 27.5% (Appendix 9.4). This result may be a potential outlier as there was no significant difference in cell death between drug J treated and control cells.

### 5.3.1 Calcium Ionophore A23187

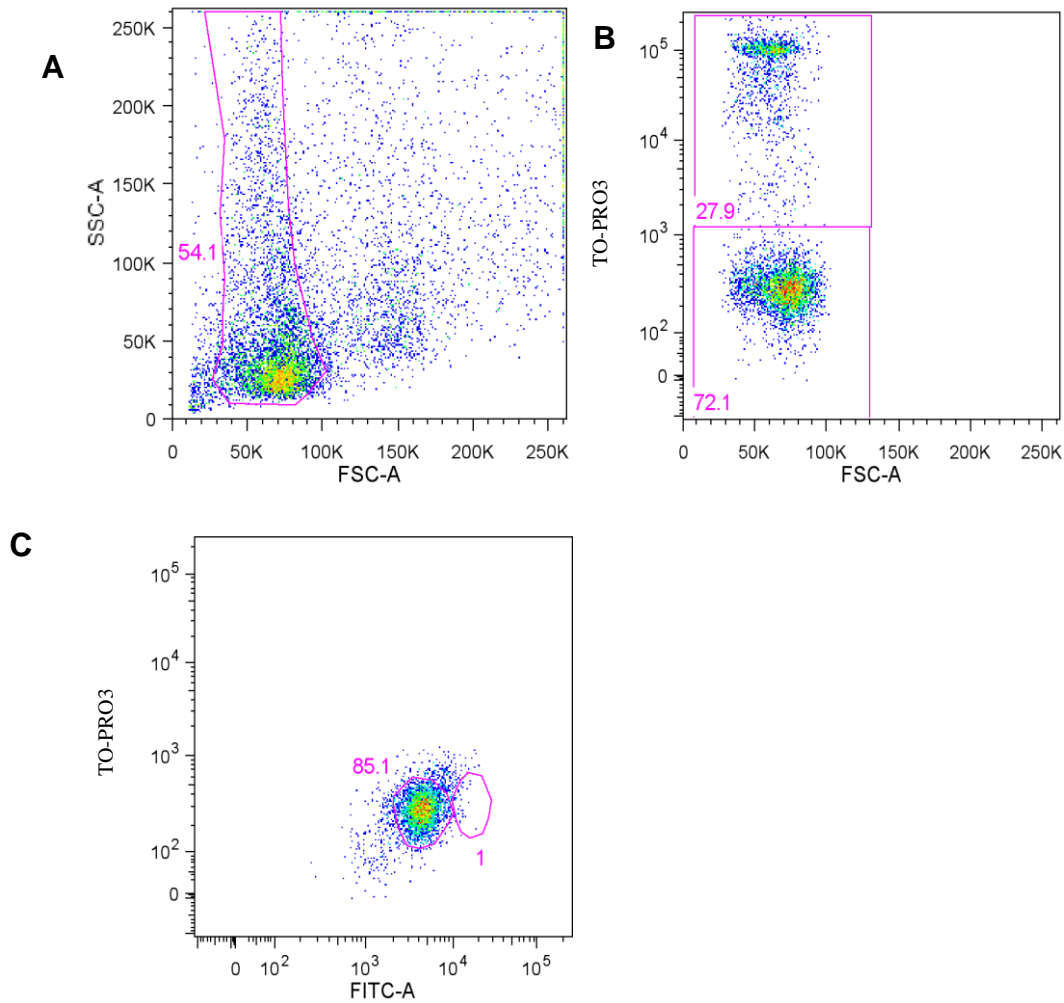


**Figure 5.1: Flow cytometry analysis after Donor 226 cells were incubated with A23187 for 60 minutes.** **A:** FSC plotted against SSC: gated region shows the population of single cells, 65.9%, from analysis of 10,000 events. **B:** Single cells: the population of single cells gated from the whole cell population were plotted as FSC against TO-PRO3 to determine the percentage of live and dead cells in the population. 76.6% of single cells are live after 60 minutes exposure to A23187 and 23.4% are dead. **C:** Acrosome and non-acrosome reacted cells: of the population of gated live cells, these were plotted as FITC-A against TOPRO-3 to determine two cell populations; non-acrosome reacted, 53.6% and acrosome reacted, 38.5%. TO-PRO3 and FITC-A are measured in wavelengths of light (nm).

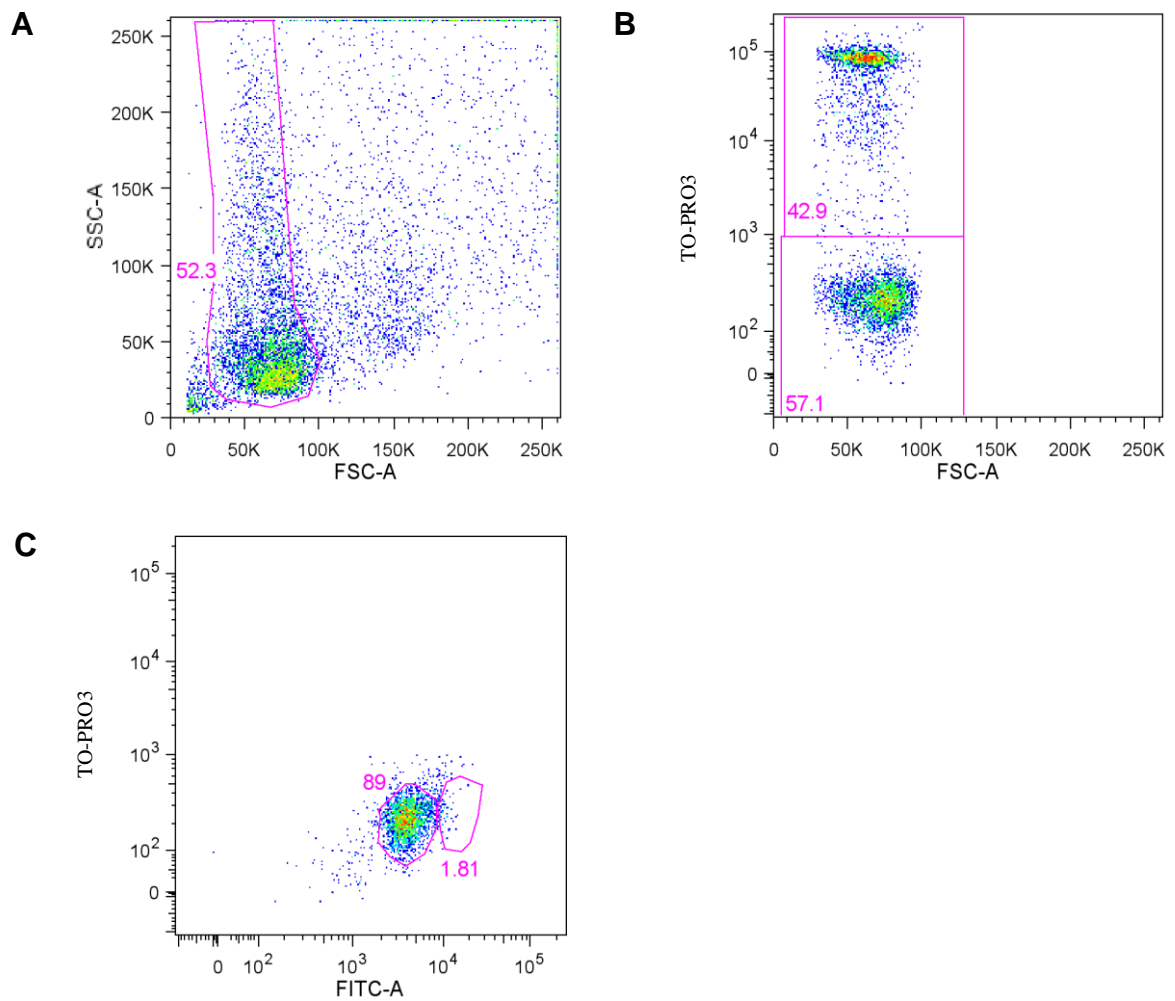


**Figure 5.2: Flow cytometry analysis after Donor 237 sperm were incubated with A23187 for 60 minutes.** **A:** FSC plotted against SSC: the gated region shows the population are single cells, 73.1% from analysis of 10,000 events analysed. **B:** Single cells: the population of single cells gated from the whole cell population were plotted as FSC against TO-PRO3 to determine the percentage of live and dead cells in the population. 66.3% of single cells are live after 60 minutes exposure to A23187 and 33.7% are dead. **C:** Acrosome and non-acrosome reacted cells: of the population of gated live cells, these were plotted as FITC-A against TO-PRO3 to determine two cell populations; non-acrosome reacted, 48.4% and acrosome reacted, 46.7%. TO-PRO3 and FITC-A are measured in wavelengths of light (nm).

### 5.3.2 DMSO



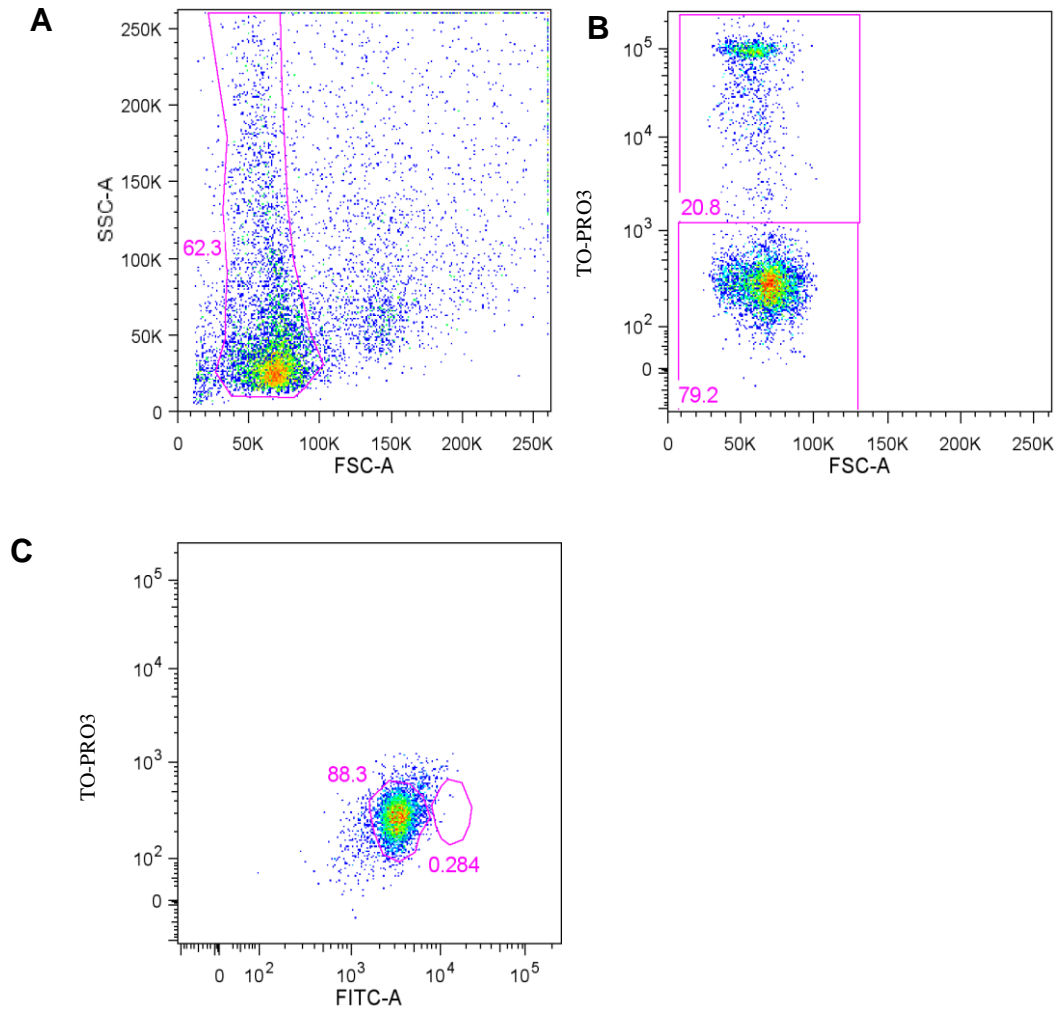
**Figure 5.3: Flow cytometry analysis after Donor 226 sperm were incubated with DMSO for 60 minutes.** **A:** FSC plotted against SSC: the gated population of single cells, 54.1% of the population of 10,000 events analysed. **B:** Single cells: the population of single cells gated from the whole cell population were plotted as FSC against TO-PRO3 to determine the percentage of live and dead cells in the sperm cell population. 72.1% of the single cells were live after 60 minutes exposure to DMSO and 27.9% were dead. **C:** Acrosome and non-acrosome reacted cells: of the population of gated live cells, these were plotted as FITC-A against TO-PRO3 to determine two cell populations; non-acrosome reacted, 85.1% and acrosome reacted, 1%. TO-PRO3 and FITC-A are measured in wavelengths of light (nm).



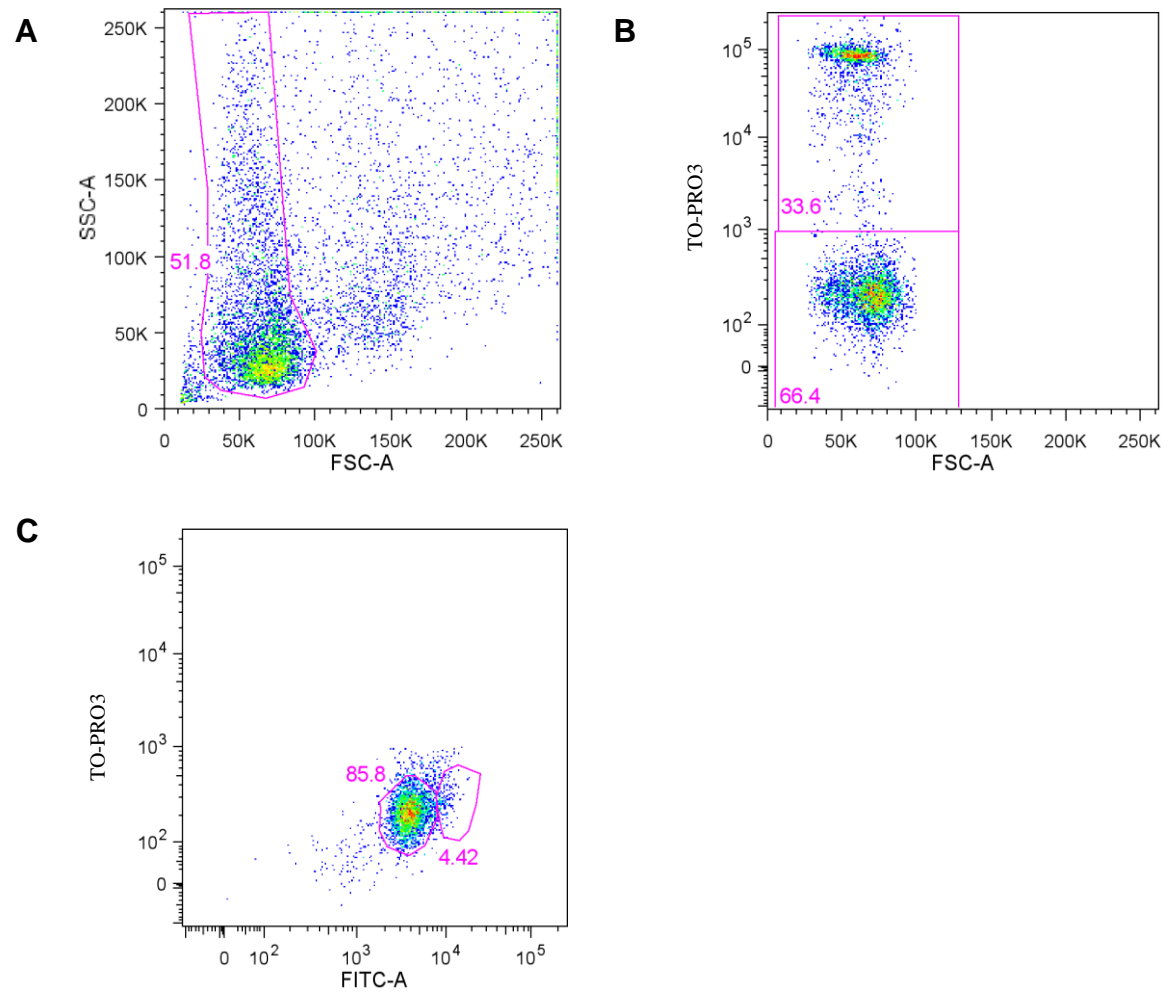
**Figure 5.4: Flow cytometry analysis after Donor 237 sperm were incubated with DMSO for 60 minutes.** **A:** FSC plotted against SSC: the gated population of single cells, 52.3% of the population of 10,000 events analysed. **B:** Single cells: the population of single cells gated from the whole cell population were plotted as FSC against TO-PRO3 to determine the percentage of live and dead cells in the sperm cell population. 57.1% of the single cells were live after 60 minutes exposure to DMSO and 42.9% were dead. **C:** Acrosome and non-acrosome reacted cells: of the population of gated live cells, these were plotted as FITC-A against TO-PRO3 to determine two cell populations; non-acrosome reacted, 89%, and acrosome reacted, 1.81%. TO-PRO3 and FITC-A are measured in wavelengths of light (nm).



### 5.3.3 Ethanol

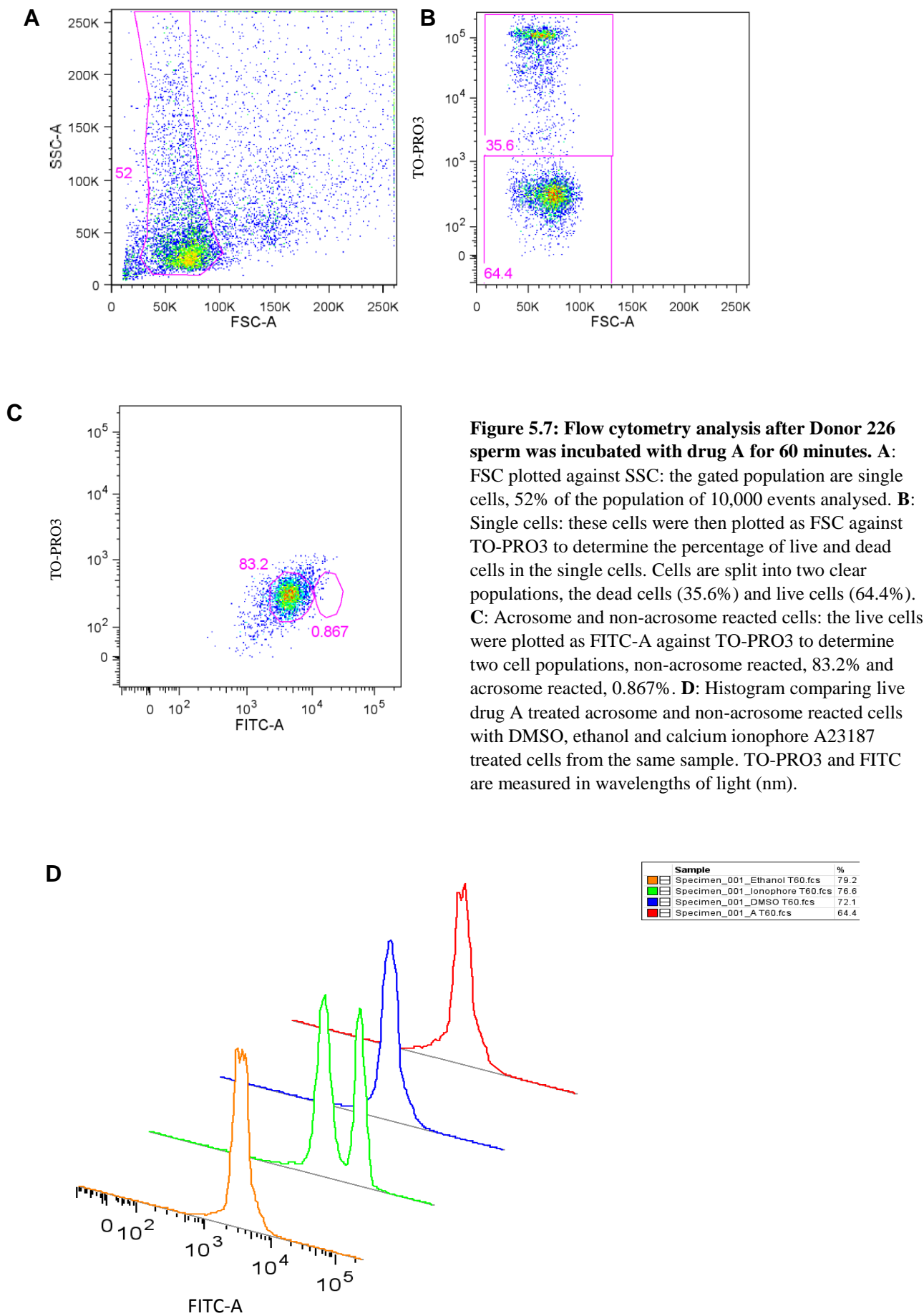


**Figure 5.5: Flow cytometry analysis after Donor 226 sperm were incubated with ethanol for 60 minutes.** **A:** FSC plotted against SSC: the gated population of single cells, 62.3% of the population of 10,000 events analysed. **B:** Single cells: the population of single cells gated from the whole cell population were plotted as FSC against TO-PRO3 to determine the percentage of live and dead cells in the sperm cell population. 79.2% of the single cells were live after 60 minutes exposure to ethanol and 20.8% were dead. **C:** Acrosome and non-acrosome reacted cells: of the population of gated live cells, these were plotted as FITC-A against TO-PRO3 to determine two cell populations; non-acrosome reacted, 88.3% and acrosome reacted, 0.284%. TO-PRO3 and FITC-A are measured in wavelengths of light (nm).



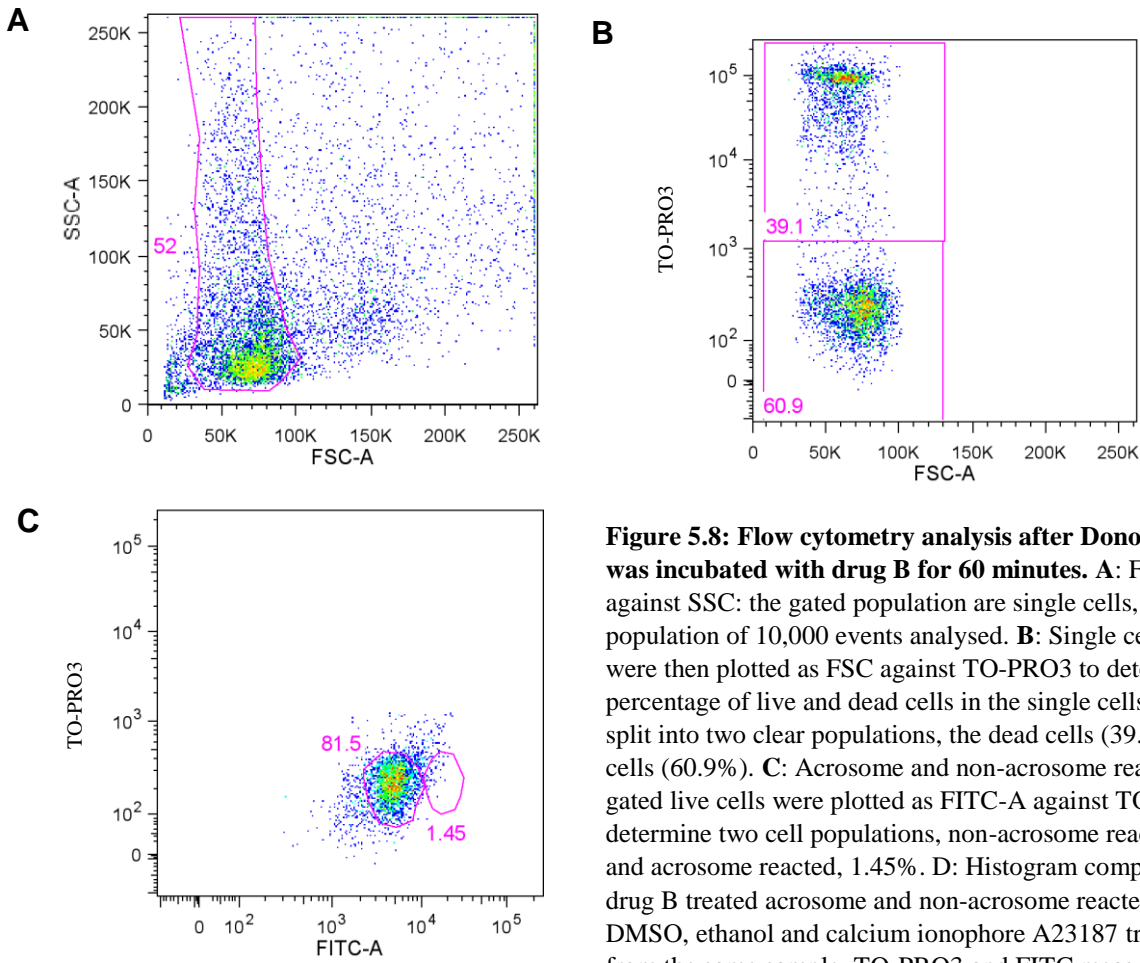
**Figure 5.6: Flow cytometry analysis after Donor 237 sperm were incubated with ethanol for 60 minutes.** **A:** FSC plotted against SSC: the gated population of single cells, 51.8% of the population of 10,000 events analysed. **B:** Single cells: the population of single cells gated from the whole cell population were plotted as FSC against TO-PRO3 to determine the percentage of live and dead cells in the sperm cell population. 66.4% of the single cells were live after 60 minutes exposure to ethanol and 33.6% were dead. **C:** Acrosome and non-acrosome reacted cells: of the population of gated live cells, these were plotted as FITC-A against TO-PRO3 to determine two cell populations; non-acrosome reacted, 85.8% and acrosome reacted, 4.42%. TO-PRO3 and FITC-A are measured in wavelengths of light (nm).

5.3.4 Drug A

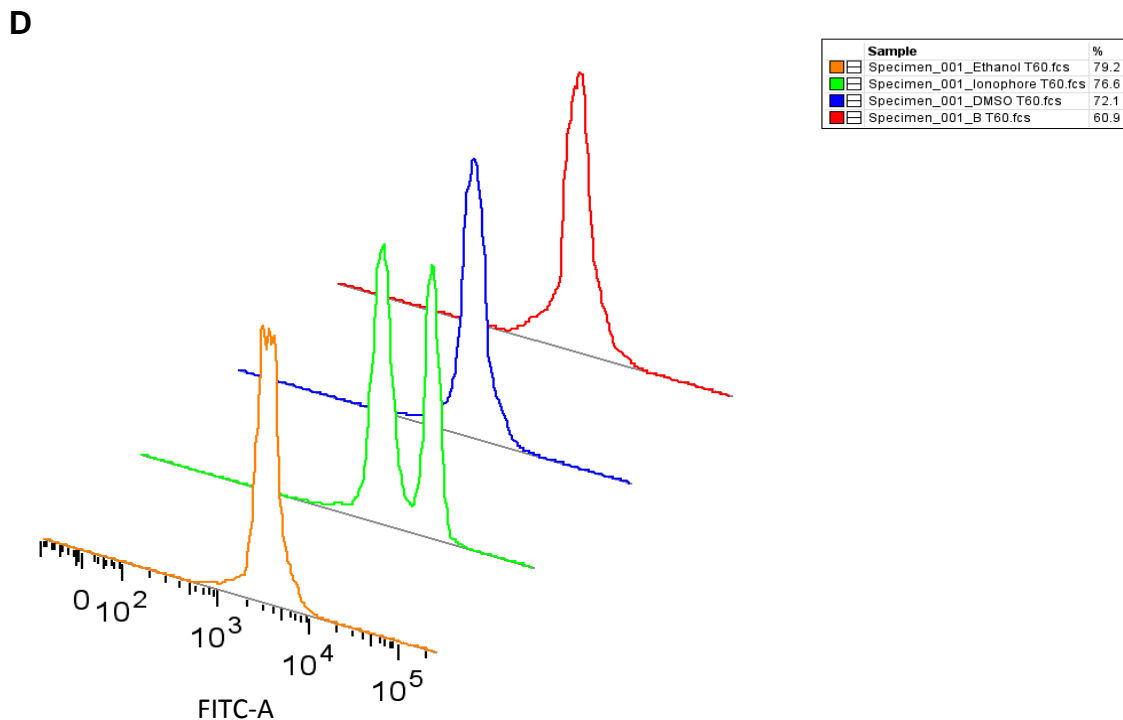


**Figure 5.7: Flow cytometry analysis after Donor 226 sperm was incubated with drug A for 60 minutes. A:** FSC plotted against SSC: the gated population are single cells, 52% of the population of 10,000 events analysed. **B:** Single cells: these cells were then plotted as FSC against TO-PRO3 to determine the percentage of live and dead cells in the single cells. Cells are split into two clear populations, the dead cells (35.6%) and live cells (64.4%). **C:** Acrosome and non-acrosome reacted cells: the live cells were plotted as FITC-A against TO-PRO3 to determine two cell populations, non-acrosome reacted, 83.2% and acrosome reacted, 0.867%. **D:** Histogram comparing live drug A treated acrosome and non-acrosome reacted cells with DMSO, ethanol and calcium ionophore A23187 treated cells from the same sample. TO-PRO3 and FITC are measured in wavelengths of light (nm).

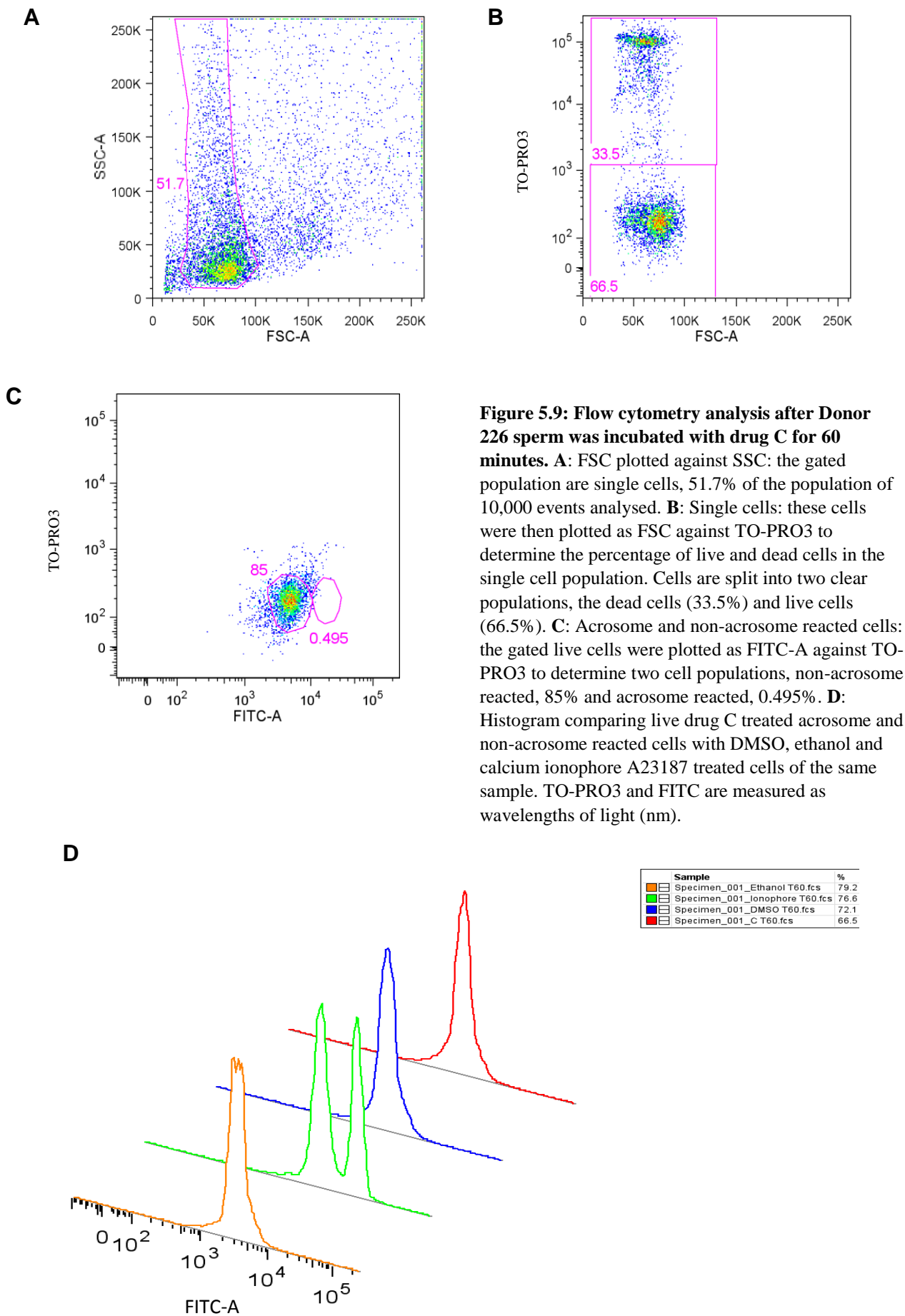
5.3.5 Drug B



**Figure 5.8: Flow cytometry analysis after Donor 226 sperm was incubated with drug B for 60 minutes.** **A:** FSC plotted against SSC: the gated population are single cells, 52% of the population of 10,000 events analysed. **B:** Single cells: these cells were then plotted as FSC against TO-PRO3 to determine the percentage of live and dead cells in the single cells. Cells are split into two clear populations, the dead cells (39.1%) and live cells (60.9%). **C:** Acrosome and non-acrosome reacted cells: the gated live cells were plotted as FITC-A against TO-PRO3 to determine two cell populations, non-acrosome reacted, 81.5% and acrosome reacted, 1.45%. **D:** Histogram comparing live drug B treated acrosome and non-acrosome reacted cells with DMSO, ethanol and calcium ionophore A23187 treated cells from the same sample. TO-PRO3 and FITC measured as wavelengths of light (nm).

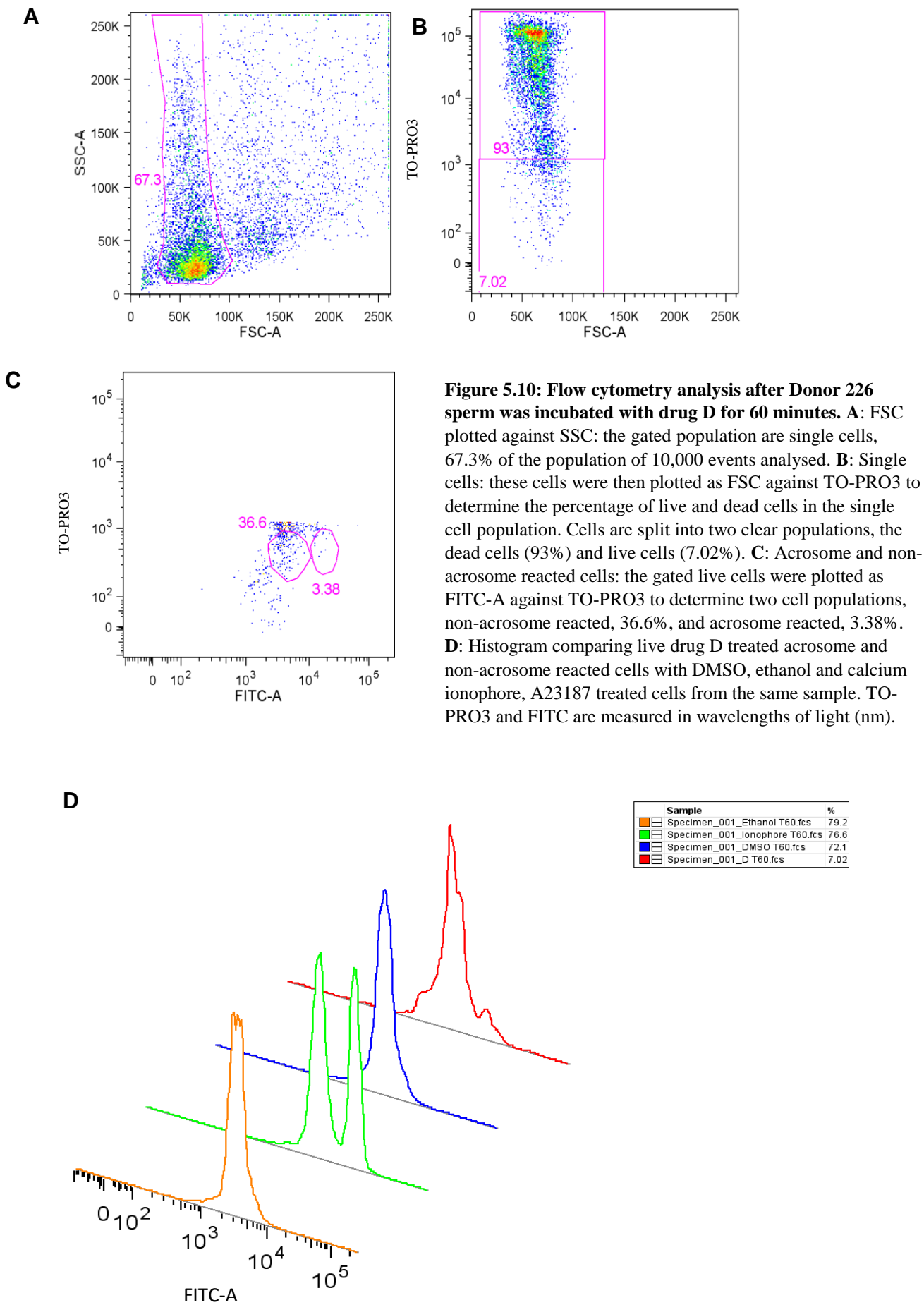


5.3.6 Drug C



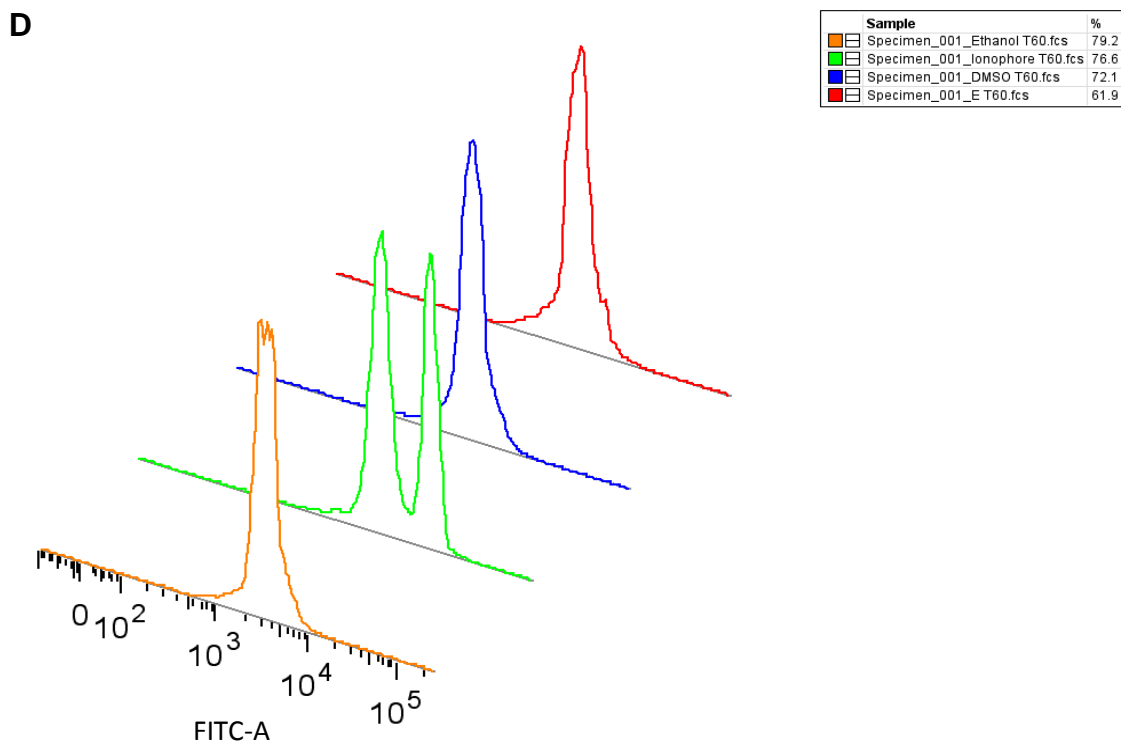
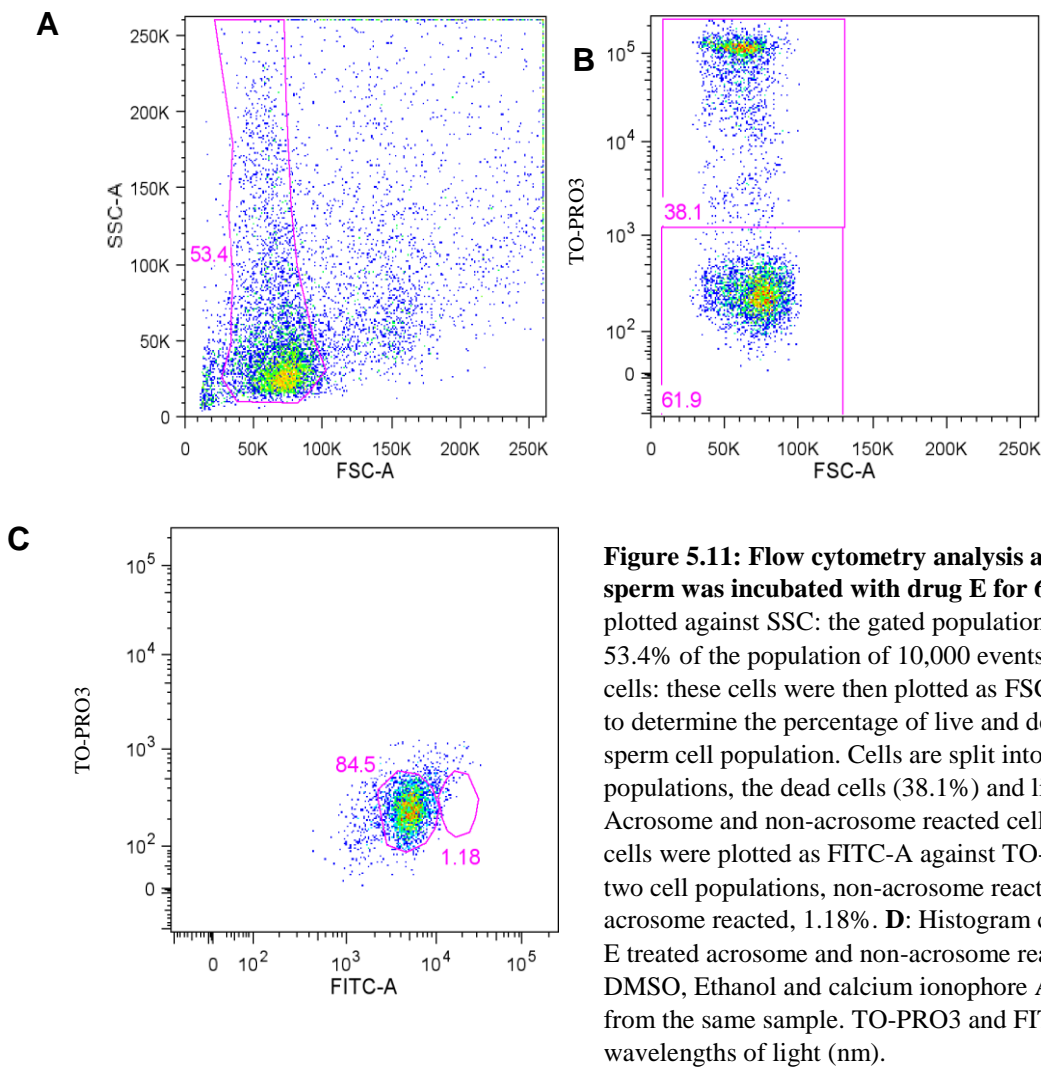
**Figure 5.9: Flow cytometry analysis after Donor 226 sperm was incubated with drug C for 60 minutes.** **A:** FSC plotted against SSC: the gated population are single cells, 51.7% of the population of 10,000 events analysed. **B:** Single cells: these cells were then plotted as FSC against TO-PRO3 to determine the percentage of live and dead cells in the single cell population. Cells are split into two clear populations, the dead cells (33.5%) and live cells (66.5%). **C:** Acrosome and non-acrosome reacted cells: the gated live cells were plotted as FITC-A against TO-PRO3 to determine two cell populations, non-acrosome reacted, 85% and acrosome reacted, 0.495%. **D:** Histogram comparing live drug C treated acrosome and non-acrosome reacted cells with DMSO, ethanol and calcium ionophore A23187 treated cells of the same sample. TO-PRO3 and FITC are measured as wavelengths of light (nm).

5.3.7 Drug D

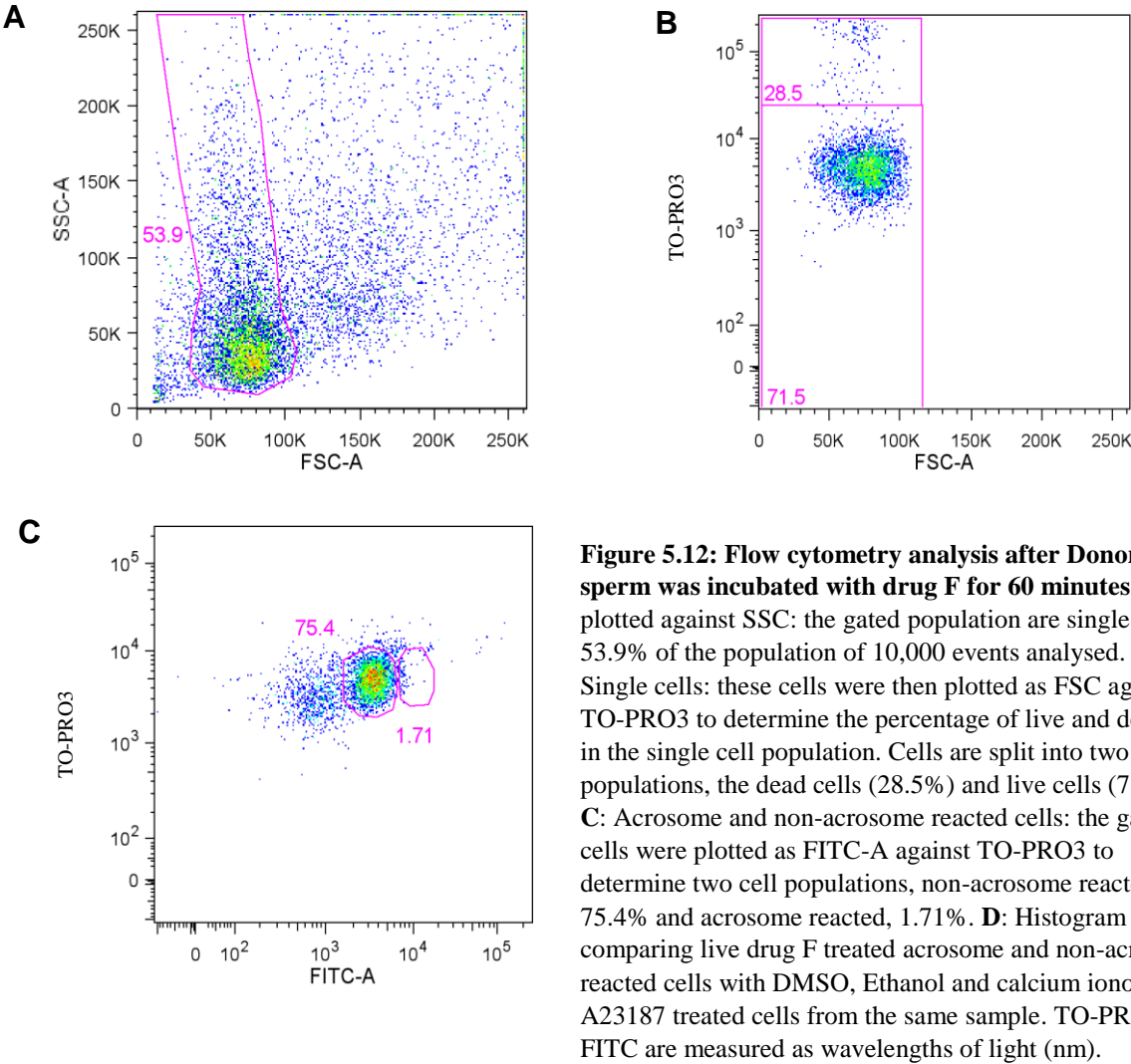


**Figure 5.10: Flow cytometry analysis after Donor 226 sperm was incubated with drug D for 60 minutes.** **A:** FSC plotted against SSC: the gated population are single cells, 67.3% of the population of 10,000 events analysed. **B:** Single cells: these cells were then plotted as FSC against TO-PRO3 to determine the percentage of live and dead cells in the single cell population. Cells are split into two clear populations, the dead cells (93%) and live cells (7.02%). **C:** Acrosome and non-acrosome reacted cells: the gated live cells were plotted as FITC-A against TO-PRO3 to determine two cell populations, non-acrosome reacted, 36.6%, and acrosome reacted, 3.38%. **D:** Histogram comparing live drug D treated acrosome and non-acrosome reacted cells with DMSO, ethanol and calcium ionophore, A23187 treated cells from the same sample. TO-PRO3 and FITC are measured in wavelengths of light (nm).

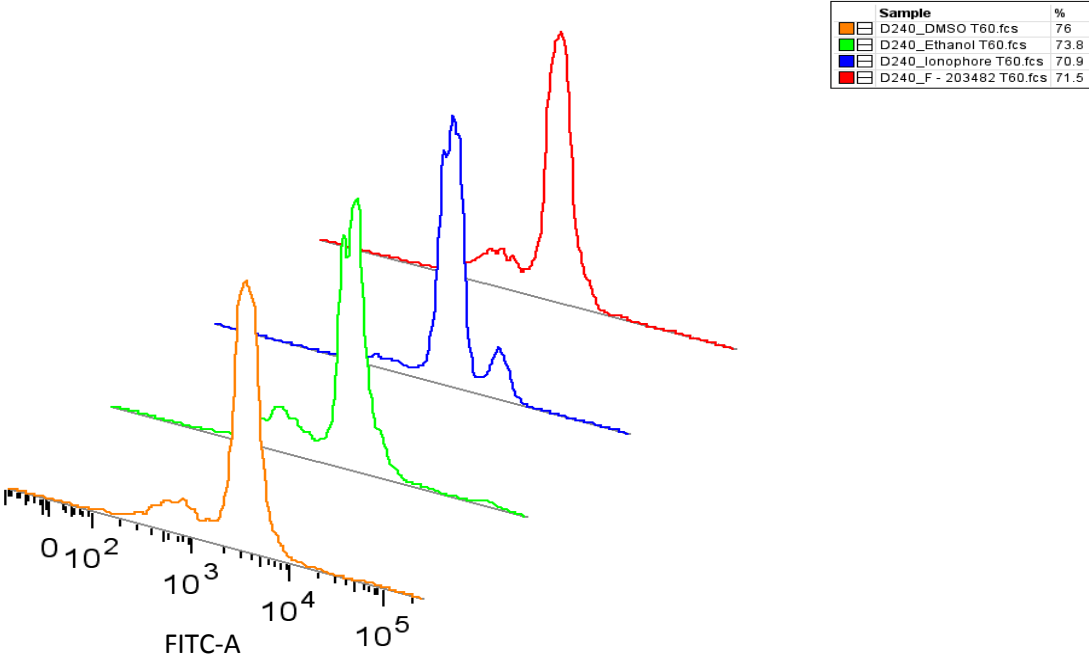
5.3.8 Drug E



5.3.9 Drug F

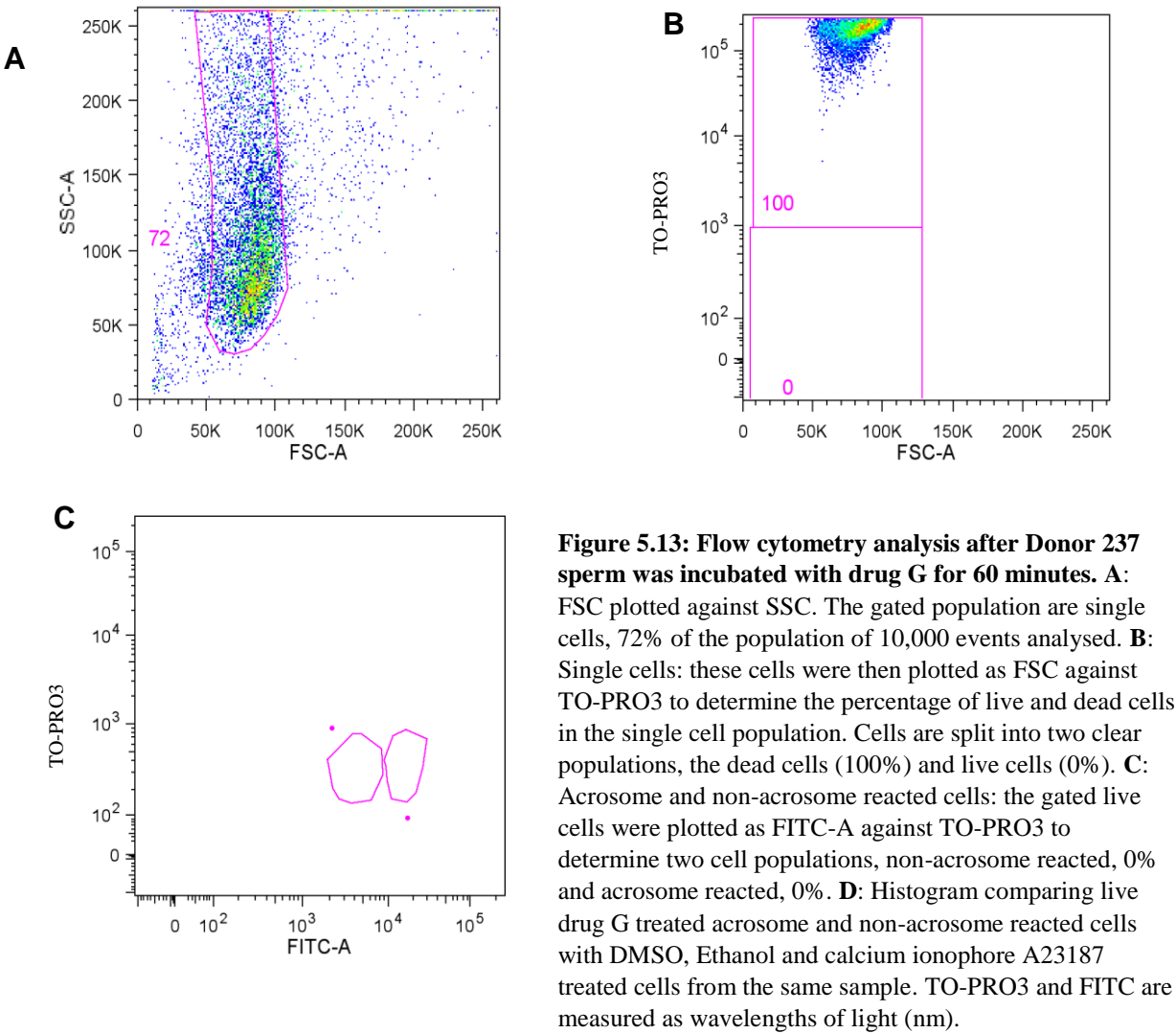


**D**



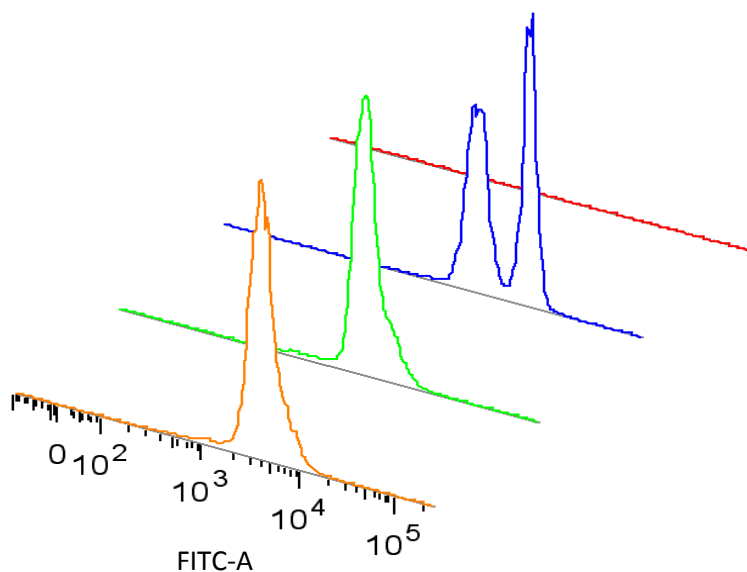


5.3.10 Drug G

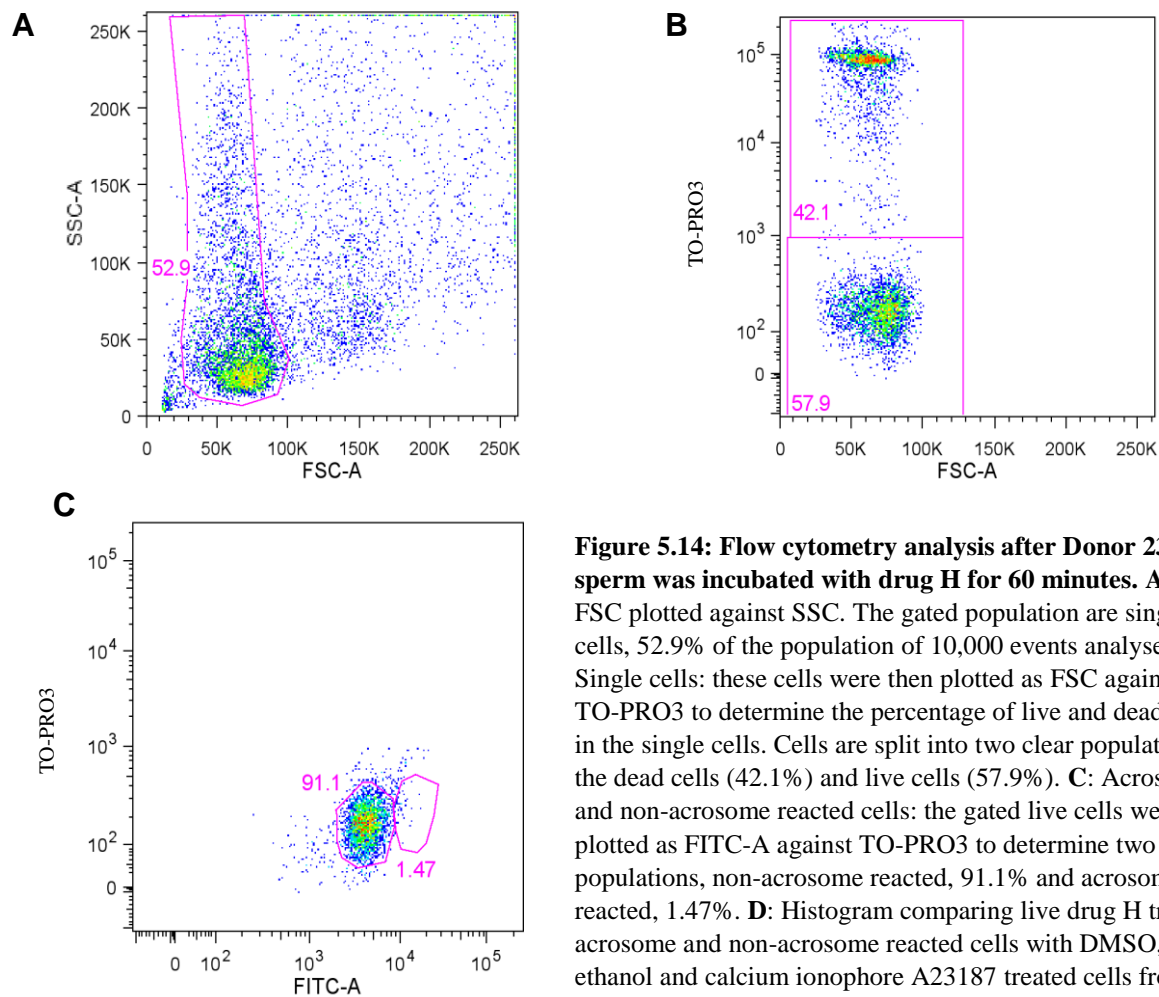


**D**

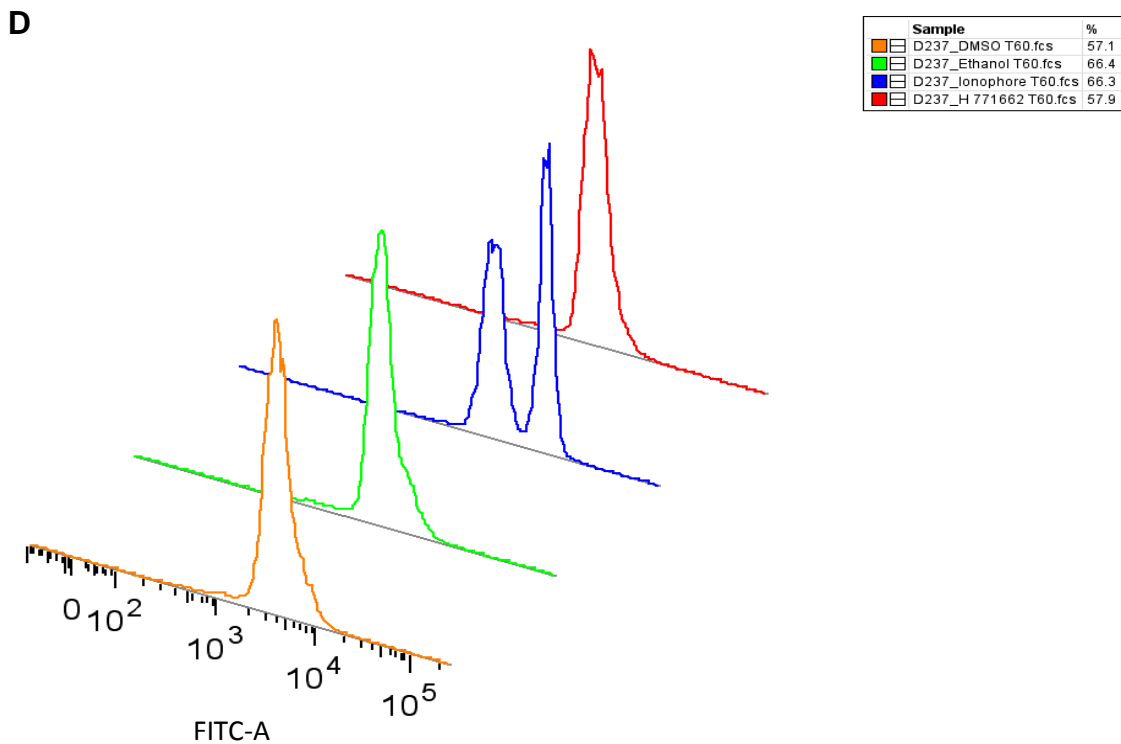
Sample	%
D237_DMSO T60.fcs	57.1
D237_Ethanol T60.fcs	66.4
D237_Ionophore T60.fcs	66.3
D237_G 203517 T60.fcs	0



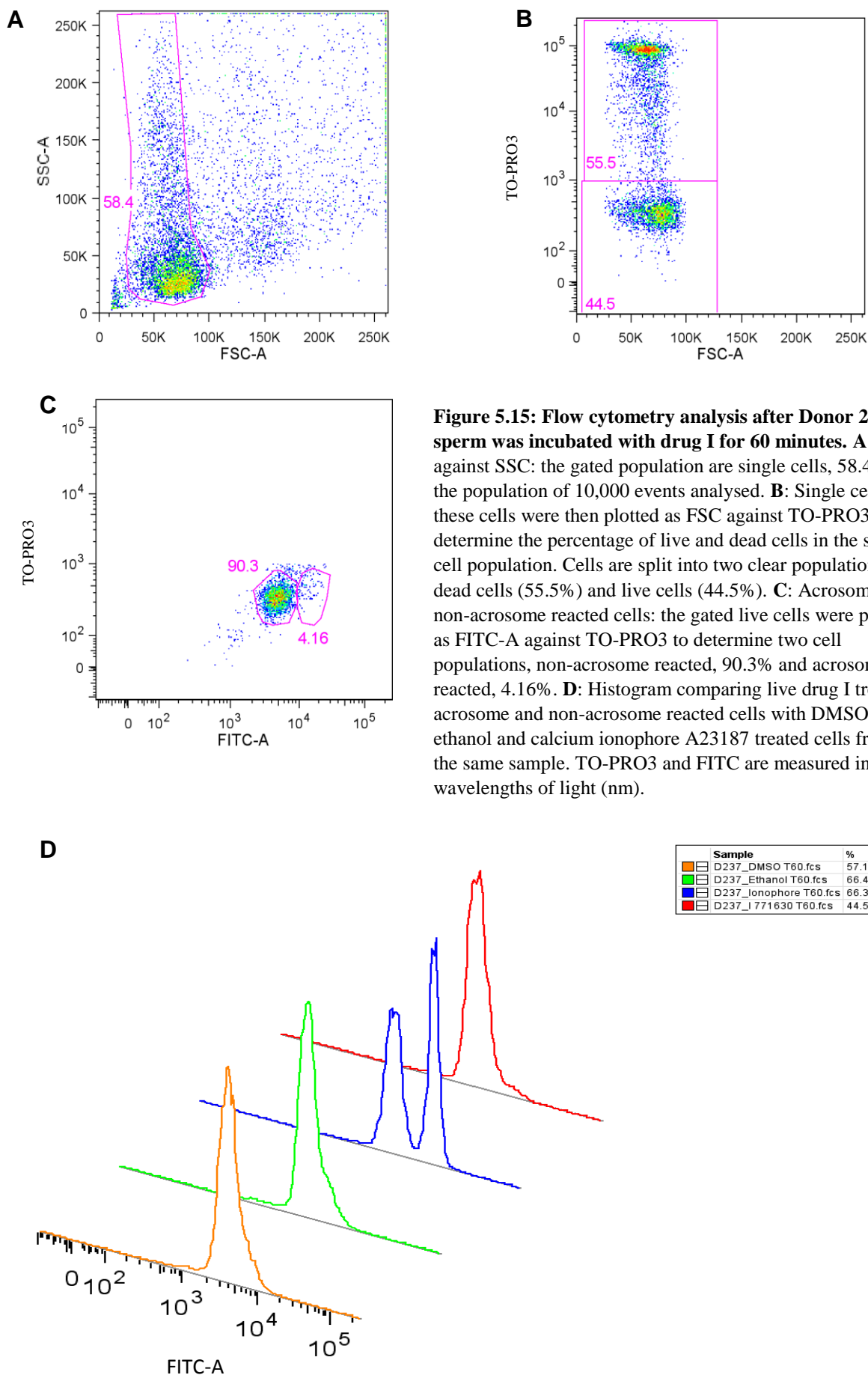
5.3.11 Drug H



**Figure 5.14: Flow cytometry analysis after Donor 237 sperm was incubated with drug H for 60 minutes. A:** FSC plotted against SSC. The gated population are single cells, 52.9% of the population of 10,000 events analysed. **B:** Single cells: these cells were then plotted as FSC against TO-PRO3 to determine the percentage of live and dead cells in the single cells. Cells are split into two clear populations, the dead cells (42.1%) and live cells (57.9%). **C:** Acrosome and non-acrosome reacted cells: the gated live cells were plotted as FITC-A against TO-PRO3 to determine two cell populations, non-acrosome reacted, 91.1% and acrosome reacted, 1.47%. **D:** Histogram comparing live drug H treated acrosome and non-acrosome reacted cells with DMSO, ethanol and calcium ionophore A23187 treated cells from the same sample. TO-PRO3 and FITC are measured as wavelengths of light (nm).

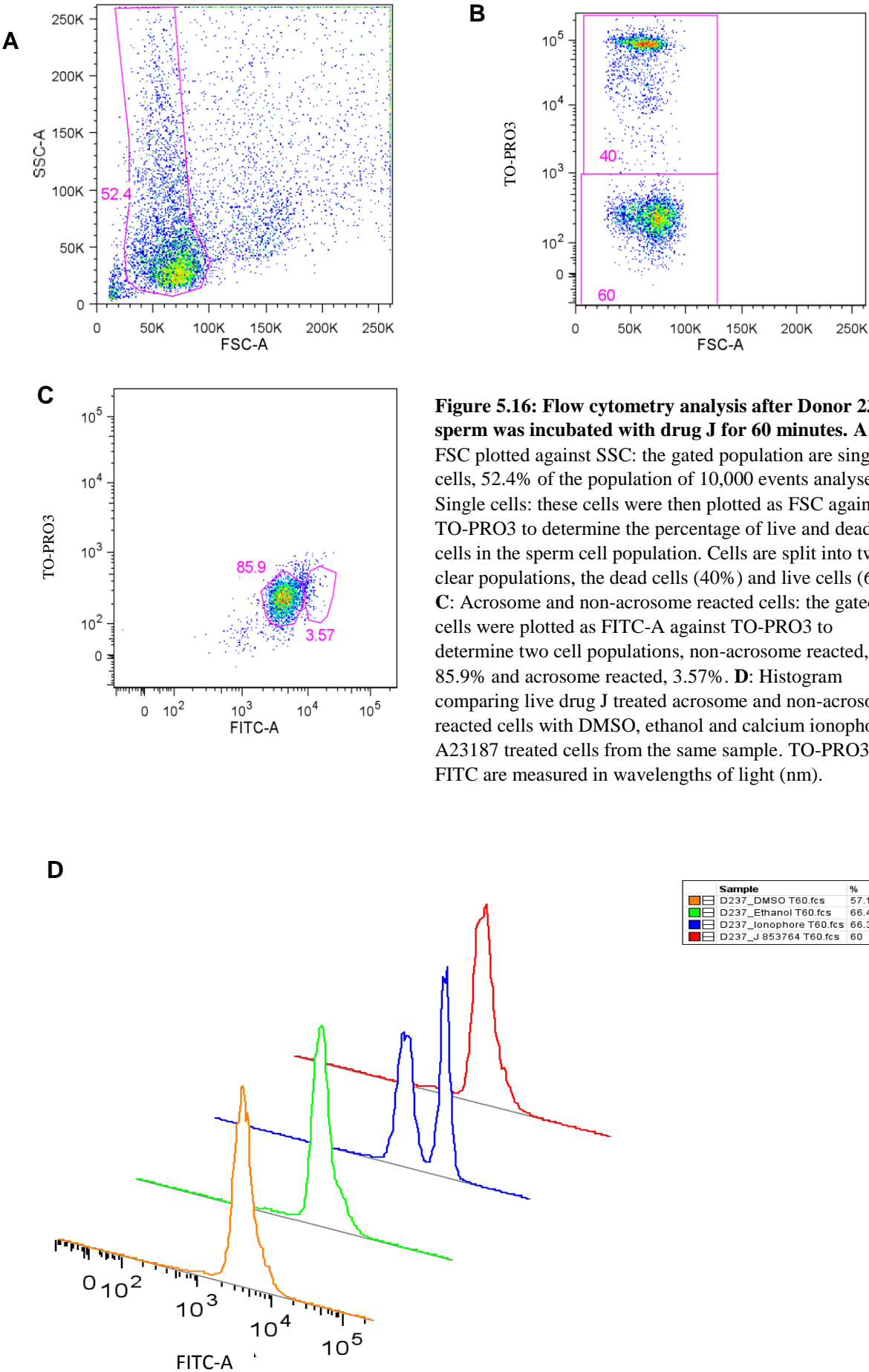


5.3.12 Drug I



**Figure 5.15: Flow cytometry analysis after Donor 237 sperm was incubated with drug I for 60 minutes.** **A:** FSC against SSC: the gated population are single cells, 58.4% of the population of 10,000 events analysed. **B:** Single cells: these cells were then plotted as FSC against TO-PRO3 to determine the percentage of live and dead cells in the sperm cell population. Cells are split into two clear populations, the dead cells (55.5%) and live cells (44.5%). **C:** Acrosome and non-acrosome reacted cells: the gated live cells were plotted as FITC-A against TO-PRO3 to determine two cell populations, non-acrosome reacted, 90.3% and acrosome reacted, 4.16%. **D:** Histogram comparing live drug I treated acrosome and non-acrosome reacted cells with DMSO, ethanol and calcium ionophore A23187 treated cells from the same sample. TO-PRO3 and FITC are measured in wavelengths of light (nm).

5.3.13 Drug J



**Figure 5.16: Flow cytometry analysis after Donor 237 sperm was incubated with drug J for 60 minutes. A:** FSC plotted against SSC: the gated population are single cells, 52.4% of the population of 10,000 events analysed. **B:** Single cells: these cells were then plotted as FSC against TO-PRO3 to determine the percentage of live and dead cells in the sperm cell population. Cells are split into two clear populations, the dead cells (40%) and live cells (60%). **C:** Acrosome and non-acrosome reacted cells: the gated live cells were plotted as FITC-A against TO-PRO3 to determine two cell populations, non-acrosome reacted, 85.9% and acrosome reacted, 3.57%. **D:** Histogram comparing live drug J treated acrosome and non-acrosome reacted cells with DMSO, ethanol and calcium ionophore A23187 treated cells from the same sample. TO-PRO3 and FITC are measured in wavelengths of light (nm).

## 5.4 Discussion

All 10 compounds were screened on three different donor samples on three separate occasions. None of the compounds showed increased induction of AR, using FCM analysis.

FCM is an experimental approach that enables the simultaneous measurement of multiple fluorescence and light scatter as cells flow quickly through a sensing area. This is induced by the illumination of these single cells, or microscopic particles in a cell suspension, by a laser (Shapiro, 1995). The advantages of using the technique to detect fluorescent cell types are the ability to measure large numbers of cells in a test sample, in an objective, rapid and reproducible manner (Dominguez-Fandos et al., 2007; Muratori et al., 2008). Some flow cytometers allow for the physical sorting and separation of cells according to their properties. FCM allows numerous biological parameters to be quantified simultaneously from single cell particles, with thousands of cells – ‘events’ - being analysed per second. This system allows the study of large heterogeneous cell populations by analysing the biometric properties of individual cells (O’Connor et al., 2001). This is a huge advantage for spermatozoa sample analysis as it is known that these cells have distinct variations within individual samples.

The use of live cells, such as motile prepared spermatozoa, allows the investigation of many biochemical parameters in minimally altered intracellular environments, along with physiologically accurate extracellular conditions (O’Connor et al., 2001).

Traditional semen analysis involves the evaluation of percentage motile spermatozoa, morphological assessments of spermatozoa and the concentration in a sample, either subjectively or using a computer assisted microscope. However, manual semen analysis is thought to not give an accurate representation of spermatozoa fertilising capacity *in-vitro* (Rodriguez-Martinez, 2003; Amann, 2005). FCM can also be used in fertility

diagnostics of semen samples in fertility laboratories as it gives an accurate, objective, rapid and inexpensive prediction of the fertility of a sample. The discovery of a wide variety of fluorochromes and compounds conjugated to fluorescent probes has allowed the use of FCM as a widespread analysis of semen quality at a biochemical, ultrastructural and functional level, and can be used accurately and rapidly to measure spermatozoa characteristics on a large scale (Gillan et al., 2005). FCM can be applied to semen evaluation to analyse various functions such as cell viability, acrosomal integrity, mitochondrial function, capacitation status, membrane fluidity and DNA status (Gillan et al., 2005). The high numbers of spermatozoa being analysed by FCM compared to regular semen analysis, with an average of 10,000 cells being analysed at each sampling interval, is another advantage of the technique (Gillan et al., 2005).

#### 5.4.1 A23187 effects and variation

Although variable between samples, the positive control (A23187; calcium ionophore) induced the AR in all donor spermatozoa. One sample showed as high as 46.7% AR cells. This is in keeping with other published studies, which indicate a variation in response to calcium ionophore A23187. Using real-time fluorescent microscopy, another study indicated that when exposed to A23187, the AR was induced in approximately 50% of spermatozoa (Harper et al., 2008). Similarly, Pitcherit-Marchenay et al., (2004) found that after incubation with A21387 for 45 minutes, the AR was induced in approximately 53% of spermatozoa using the fixed cell method. Aitken et al., (1993) found that, after a dose response study, 10 $\mu$ M ionophore induced the highest percentage of AR cells with 80% having lost their acrosome. And although they were immotile, according to a hypoosmotic swell test, they had suffered no loss of structural integrity.

There was a wide variation between donors in their response to calcium ionophore A23187 in this study, (10% – 46.7% AR). Although all 6 donors responded, this variation would suggest some spermatozoa may be more susceptible to induction of the AR than others. Semen samples are known to be a heterogeneous population and show massive variation between donors in many aspects such as concentration, motility, morphology and HA therefore it is not surprising that there is significant biological variation in their response to treatment with compounds to induce biological responses.

In this study, similar to Zoppino et al., four cell populations were evident after analysis; unreacted dead cells, reacted dead cells, unreacted live cells, and reacted live cells. After 1 hour of incubation with the calcium ionophore, reacted live cells increased to around 60%. The population of dead cells remained under 8% (Zoppino et al., 2012). During FCM, spermatozoa undergo various stresses therefore there are many reasons, out with biological variation, that could account for variations in spermatozoa populations; instrument factors influencing the resolution of measurement; fluidic instability, laser noise, electronic and photodetector noise, light collection efficiency, and acoustic vibration (Sharpe & Evans, 2009).

#### 5.4.2 Cell Death

A cell death marker (TO-PRO3) was used to study spermatozoa viability to determine if any of the compounds were having detrimental effects on cell survival after 1 hour incubation. FCM can also be used to analyse cell death by apoptosis or necrosis (Darzynkiewicz et al., 1997; Banki, K., et al. 1999) by differentiating between live and dead cells by the ability of their intact plasma membranes to inhibit stain entry into the cell (Mayer et al., 1951; Hackett & Macpherson, 1965). TO-PRO3 stain was used in this experiment to penetrate the cell membrane and stain the nuclei of dying cells.

Two compounds, D and G, indicated severe detrimental effects on donor spermatozoa (n=3). Compound G caused 100% of all cells to die after 1 hour incubation ( $P<0.0001$ ), and compound D caused between 85.4%-95.7% cells to die ( $P<0.0001$ ). Drug D, Trifluoperazine, is an anti-anxiety drug used to treat schizophrenia. As described previously (Chapter 5.4.3), Trifluoperazine blocks dopamine receptors which may have caused severe detrimental effects by inhibiting tyrosine phosphorylation and inducing cell death.

Drug G, is known as MK886, an inhibitor of 5-lipoxygenase-activating protein (FLAP). Little is known about the 5-lipoxygenase pathway, although it is involved in the production of the eicosanoid inflammatory mediators (leukotrienes) in leucocytes. MK-886 selectively binds to the FLAP, an integral membrane protein that may facilitate substrate transfer to 5-lipoxygenase (Mancini et al., 1993). MK886 is known to suppress leukotriene biosynthesis via leukotriene C<sub>4</sub> synthase (Lam et al., 1994) and impairs prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) formation via direct interference with microsomal PGE<sub>2</sub> synthase (Claveau et al., 2003). MK886 is also known to have antiproliferative effects and induces apoptosis in various cancer cell lines, but only at concentrations (1-50  $\mu$ M) which are much higher than those needed to inhibit leukotriene synthesis (Anderson et al., 1995; Datta et al., 1998; Ghosh and Myers, 1998; Gugliucci et al., 2002). The antiproliferative effects of MK886 are seen in cells that do not express 5-lipoxygenase (Datta et al., 1998; Stika et al., 2006), and MK-886 induced apoptosis independent to 5-lipoxygenase and FLAP (Datta et al., 1999; Wu et al., 2003), supporting the existence of additional related targets for MK886. Spermatozoa are not known to express 5-lipoxygenase, which may indicate a possible reason why MK886 induced severe cell death after 1 hour exposure.



A23187, ethanol, and compounds A, B, C, E, and F all had variable cell death after 1 hour incubation, but in each donor sample, and for each compound the percentages were all below 40%. The sample with the lowest percentage of cell death was 17%, similar to the Zoppino et al., study after 25 minutes of observation using time lapse imaging (Zoppino et al., 2012).

Semen samples have a very heterogeneous population. There is great variation between ejaculates and within ejaculates, as well as between individual spermatozoa and reactions to different treatments and experiments. When analysing the 80% spermatozoa fraction, which was used in the FCM experiments, after DGC and before incubation with compounds, one indication of sample health is percentage motility. Percentage motility in all six donors was between 75%-90% showing a very high level of cell viability at the beginning of the experiment. The process of FCM and incubation with the compounds is expected to have a slight detrimental effect on cell health as a result of dilution, high pressure in the flow cytometer and exposition to the laser (Van Dilla, 1977). Regarding this, a cut off value of 60% live cells was used to determine if any compound was having detrimental effects on cell viability.

DMSO, compound H and J all had two donor samples within the lower limits (15.9% - 29.9%), but 1 donor sample (D237) indicated cell death of these three compounds to be above 40% (42.9%, 42.1%, 40%). These are borderline results and it is possible that this donor could be regarded as an outlier. Also, the cells may not have been healthy due to faults in the handling and preparation of the cells, changes in temperatures when moving spermatozoa from Ninewells hospital to the Dundee DDU, cells were centrifuged multiple times to change media, and the compounds and FCM process and handling could be affecting the results. Also it has been reported that, when compared with fluorescent microscopy, FCM produces values of spermatozoa damage which are 2.6 times higher (Dominguez-Fandos et al., 2007).

Drug I indicated an average cell death of above 40% (36.1% - 55.5%), and the compound was found to have slight significant detrimental effects ( $P=0.0119$ ) on cell viability. Drug I is Nebivolol, a beta adrenoreceptor blocker. Alpha<sub>2A</sub> and beta 1 and 2 adrenergic receptors are located on the spermatozoa head, particularly the acrosomal cap, and the flagellum in both mouse and human spermatozoa (Adeoya-Osiguwa et al., 2006). It is known that propranolol (a beta-blocker) can inhibit human spermatozoa motility in a concentration dependent manner (Hong et al., 1981). Other studies on the effects of beta-blockers on spermatozoa motility found that spermatozoa immobilization is an outcome of the local anaesthetic effect of beta-adrenoreceptor blockers. It was thought that the site of action was the cell membrane (Peterson & Freund, 1973; Tamblyn & First, 1977). If these drugs were administered systematically, the concentrations of the drug that crossed the blood-testis barrier would be too small to cause an effect on spermatozoa motility, but local application of these drugs, for a contraceptive target, would potentially be effective (Hong & Turner, 1982). This reduction in motility could correlate with increased cell death, which would explain the decreased cell viability seen in this experiment.

#### 5.4.3 Reliability of fluorescent tags

In the Zoppino et al study, after incubation and analysis by FCM, the cells were sorted by the fluorescent activated cell sorter (FACS) and analysed by confocal microscopy. In the live reacted cell population, almost 100% of these cells showed a clear FITC label in the acrosome region that indicates that these spermatozoa were sorted by acrosome staining and not by unspecific labelling. The unreacted live cells showed no FITC labelling. None of the live cells showed any PI staining. This illustrates and gives conformation that the fluorescent probes are specific and the fluorescent images and analysis are accurate to that specific probe (Zoppino et al., 2012).

When comparing donors and compounds, the fluorescent wavelengths of the majority of live cells appeared to vary. The compounds and control treated cells, particularly the ionophore, may be quite hard on the cell membranes, causing damage or rupture and increasing the permeability of the cells. The graphs indicate changes in TOPRO-3 fluorescent wavelengths between donors and experiments, which is due to membranes becoming more permeable in treated cells allowing more dye to move in and so the signal is higher – cells become more ‘leaky’ after treatment.

None of the 10 compounds from the Selleck NIH prescribables library of the DDU induced the AR. This is a positive result for the compounds that increased motility parameters, as this represents supportive evidence to encourage use in a clinical setting.

## **CHAPTER 6**

# **Patient Sample Screen of Motility Inducing Compounds**

## Chapter 6 - Patient Sample Screen on Motility Inducing Compounds

### 6.1 Introduction

Translational research, in the medical research context, aims to apply findings from basic science to enhance health and wellbeing. In the context of male infertility, translational research can be used to harness the knowledge from basic spermatozoa physiology understanding to produce new drugs that target and enhance spermatozoa motility and function. The clinical end point is the production of a promising new drug treatment which enhances spermatozoa fertilisation potential, and so can be used in ART clinical practise. The fundamental aim of any drug discovery project is to translate positive results from experimental drug screening to clinical leads for formal phased trials. Through the use of patient samples this explores the next stage in experimental drug screening, making the results clinically relevant.

Using IVF and ICSI patient samples from the ACU in Ninewells Hospital Dundee to support previously described motility results using donor samples provides more evidence for the benefits of these compounds to be of use in clinical practise. Fertile donor samples only provide limited information on an experimental technique or the effects of a compound because the cells are healthy, with good motility and function. Therefore positive results from patient samples with spermatozoa dysfunction provides a lot more information, and would help to support the hypothesis that compounds that increase spermatozoa motility parameters will allow for greater fertilisation potential of spermatozoa in ART.

To support this, a previous study on PDE inhibitors found that the most significant positive effects on spermatozoa were seen in those samples with the lowest concentration of motile cells (Fisch et al., 1998). If the cell motility is already high – above 70% - which is seen in samples from healthy donor males, it is less likely that a compound will induce a significant increase in cell motility because the cells are already

very high quality (Tardif et al., 2014). However, using patient samples with low cell motility to screen hit compounds should indicate a higher significant increase in motility with the compounds having a more pronounced effect, defining clearly the advantage of their use in a clinical setting.

To determine whether a patient will undergo a cycle of IVF or IUI, the Total motile count (TMC) – which is derived from semen volume, sperm concentration and sperm motility in the semen sample – is used as a guide. Patients with a TMC of 10 million or more are suitable candidates for IUI, whereas patients with less than 10 million are suggested to undergo IVF (Van Voorhis et al., 2001). Mature motility patterns are characterised as high frequency, low amplitude beats that result in progressive movement (Wald, 2008). Spermatozoa with impaired motility results in the inability of natural conception and requires expensive, invasive and sophisticated ART treatment, such as ICSI. Finding ways to improve patient spermatozoa motility in the ways described (increasing TMC and progressive or hyperactive movement) would allow the increased use of IVF and IUI, therefore reducing the need for ICSI (Wald, 2008).

A motility screen was done on a selection of IVF patient samples and an ICSI sample using compounds which induced positive motility enhancing effects on donor cells. Kremer tests on IVF samples were also undertaken to determine if the compounds enhancing effects on motility induced an increase in cell functional ability. Although the Kremer screen on donor cells was not significant, it was repeated using patient samples as their known cell dysfunction may result in the a significant positive effect that could not be induced in healthy spermatozoa. It was hypothesised that the increase in HA seen in donor spermatozoa would also be induced in patient samples, possibly with higher significance because these cells are of reduced quality.

## 6.2 Experimental Design

IVF and ICSI patient samples were produced and prepared at the ACU in Ninewells Hospital (Chapter 2.3.2). In the Ninewells ACU, males with approximately  $1 \times 10^6$  motile cells after preparation are, generally speaking, allocated to IVF (unless they have experienced previous failed or low fertilisation) and any men below this were allocated to ICSI. More IVF patient samples were used in this study because there are usually a sufficient number of surplus cells to subject to screening. Only 1 ICSI sample was examined because they usually have very low sperm count, therefore after samples are used in the clinic for treatment, there is very few cells available for research. In some cases of ICSI samples, patients have had failed fertilisation during a previous IVF cycle and so undergo ICSI in the next cycle therefore their sample will have a higher cell count, allowing more surplus cells for research, which may have been the case with this patient.

IVF patient samples were resuspended in Quinn's Advantage® fertilisation medium supplemented with human serum albumin (HSA), which supports capacitation. ICSI samples were left in 100µl SAGE® gamete buffer, which does not support capacitation. Cell number in each patient sample dictated how many drugs and varying concentrations that could be tested.

It is worth noting that IVF semen samples from patients attending the ACU are from unselected couples attending for ART and are not necessarily all men with male factor infertility.

### 6.2.1 Motility screen

Motility screen on patient samples were undertaken as previous described (Chapter 2.7). Patient samples in this study were screened using drugs C and F, which were selected after the motility screen on donor spermatozoa as they both showed significant

increases in motility parameters – specifically HA - in the 80% cell fraction (Chapter 3). Patient cells were screened at different concentrations; 30 $\mu$ M or 100 $\mu$ M or both, depending on the cell number in each sample. Spermatozoa were analysed at 0 minutes (T0), 15 minutes (T15) and 30 minutes (T30) using CASA microscope and computer software.

#### 6.2.2 Kremer penetration test

Kremer tests were undertaken as previously described (Chapter 2.8). Kremer tubes, containing 10% MC, were placed into Eppendorf tubes which contained 100 $\mu$ l of 10M/ml spermatozoa (control), 10M/ml spermatozoa and 3.6 $\mu$ M progesterone (positive control) or 10M/ml spermatozoa and compound (C or F) – screened at 30 $\mu$ M. Cells were counted, as previously described, at 1cm and 2cm. These values for drug treated spermatozoa were compared with control and progesterone to determine any effect of compounds on patient samples.

#### 6.2.3 Statistics

T tests were done on motility results for statistical comparison of drug treated cells with controls. ANOVA (analysis of variance) tests were done on Kremer results comparing control, progesterone and drug treated samples. Tukey test was used post ANOVA to indicate statistical comparisons between groups. Statistical significance was defined by  $P < 0.05$ .



### 6.3 Results

#### 6.3.1 IVF patient motility

Of the 6 IVF patients screened with drug C 30 $\mu$ M, 3 indicated a significant increase in % HA (R1707 T0: P=0.0012; R1704 T0: P=0.0154; R1742 T0: P<0.0001 and T30: P=0.0321) (Figure 7.2; 7.4; 7.8). In 2 of the patients screened, a significant increase in % motility was observed (R1707 T0: P=0.0123; R1704 T0: P=0.0112 and T15: P=0.0487) (Figure 7.2; 7.4). There was a varied response on % progressive motile cells to drug C 30 $\mu$ M, with 2 patient samples indicating a significant decrease (R1707 T15: P=0.0321; R1709 T15: P=0.036) (Figure 7.2; 7.5) and 1 illustrating a significant increase (R1704 T0: P=0.0118 and T15: P=0.0079) (Figure 7.4). These positive responses are similar to that seen in the motility screen with around half of the donors indicating a reaction to the compound at this concentration, but the effects wear off by 30 minutes, going back to control levels.

But, on screening with drug C at a higher concentration - 100 $\mu$ M – 6 out of 8 patients indicated a significant increase in % HA when compared with control samples, with one lasting until T30 (R1678 T0: P=0.0092 and T15: P=0.0044; R1707 T0: P=0.001, T15: P=0.0029 and T30: P=0.0133; R1682 T0: P=0.0037 and T15: P=0.0002; R1709 T0: P=0.0485; R1716 T0: P=0.0043; R1742 T0: P=0.0123) (Figure 7.1; 7.2; 7.3; 7.5; 7.7; 7.8). However, after exposure of Drug C 100  $\mu$ M to IVF patient sample R1716 (Figure 7.7), the initial significant increase in % HA became a significant decrease by T30 (P=0.0044) when compared with control. An increase in HA appeared to correlate with a decrease in % progressive cells, with 7 of the 8 patients showing a significant decrease in % progression (R1678 T0: P=0.0003 and T15: P=0.0037; R1707 T0: P=0.0009, T15: P=0.0058 and T30: P=0.0278; R1682 T0: P=0.0019 and T15: P=0.0066; R1709 T0: P=0.0072 and T15: P=0.0294; R1712 T0: P=0.0011, T15: P=0.0463 and T30:

P=0.0114; R1716 T0: P=0.0003 and T15: P=0.0058; R1742 T15: P=0.0129 and T30: P=0.0267) (Figure 7.1; 7.2; 7.3; 7.5; 7.6; 7.7; 7.8). Also 4 of the IVF patients illustrated a significant decrease in % motility (R1682 T15: P=0.0491 and T30: P=0.0004; R1709 T15: P=0.0436; R1712 T0: P=0.0105, T15: P=0.0328 and T30: P=0.0131; R1742 T15: P=0.0131 and T30: P=0.033) (Figure 7.3; 7.5; 7.6; 7.8), which suggests a detrimental effect, but as seen from chapter 6 (Flow cytometry), incubation after 1 hour did not induce cell death in donor cells. Also, IVF sample R1704 (Figure 7.4) incubated with drug C 100 $\mu$ M induced a significant increase in progressive motility by T30 (P=0.0138) and a significant increase in % motility at T0 (P=0.031) and T15 (P=0.0305), suggesting positive results.

Of the 4 IVF patient samples screened with Drug F 30 $\mu$ M, 1 (R1716) indicated a significant decrease in % HA at T30 (P=0.0027) (Figure 7.7). A possible detrimental effect on % progressive and % motile cells can be observed as 3 (R1678 T30: P=0.0007; R1704 T15: P=0.0265 and T30: P=0.017; R1716 T0: P=0.0238 and T30: P=0.019) (Figure 7.1; 7.4; 7.7) and 2 (R1678 T15: P=0.0345; R1704 T15: P=0.0084 and T30: P=0.0063) (Figure 7.1; 7.4) of the 4 patients, respectively, show a significant decrease. Only 1 sample indicated a significant increase in % motility after treatment with Drug F 30 $\mu$ M (R1707 T0: P=0.0428) (Figure 7.2). This possible detrimental effect is contradictory to previous screening on donor samples, as flow cytometry did not indicate any negative effects of drug F after 1 hour incubation. On screening drug F at a higher concentration (100 $\mu$ M), all 5 patient samples indicated a significant decrease in % progressive cells (R1678 T0: P=0.01, T15: P=0.0099 and T30: P=0.044; R1707 T15: P=0.0191 and T30: P=0.0428; R1682 T0: P=0.0097; R1704 T0: P=0.0058, T15: P=0.0048 and T30: P=0.0286; R1716 T0: P=0.0105) (Figure 7.1; 7.2; 7.3; 7.4; 7.7) and 4 of the 5 patients a significant decrease in % motility (R1707 T15: P=0.0428; R1682 T0: P=0.02334 and T30: P=0.0482; R1704 T0: P=0.0123, T15: P=0.0235 and T30:

P=0.0024; R1716 T30: P=0.0426) (Figure 7.2; 7.3; 7.4; 7.7). Also at this higher concentration, 2 patient samples showed a significant decrease in % HA (R1707 T30: P=0.0486; R1716 T30: P=0.0048) (Figure 7.2; 7.7) and 1, a significant increase (R1682 T0: P=0.0273) (Figure 7.3).

### 6.3.2 ICSI patient motility

Only 1 ICSI sample was screened in this experiment, because samples that are used for ICSI usually contained very few cells and therefore a motility screen was difficult to do. The 1 sample only had enough cells to test drug C at 100 $\mu$ M. In the ICSI R1748 sample screened with Drug C 100  $\mu$ M (Figure 7.9), there was a significant decrease in % progressive movement (T0: P= 0.0048, T15: P= 0.0022, T30: P= 0.0041) and % motility (T0: P= 0.0032, T15: P= 0.0014, T30: P= 0.0004) at all time points.

### 6.3.3 IVF patient Kremer

5 IVF patient samples were incubated with drugs C and F at 30 $\mu$ M to determine the effects on spermatozoa function, which was achieved using the Kremer test. After incubation with drug C, 4 patient samples indicated no significant difference in cell number at 1 cm when compared with controls (R1724, R1725, R1734, R1770) (Appendix 9.5), and 1 showed a significant decrease (R1730: P=0.0291) (Appendix 9.5). At 2cm there was no significant difference seen in all 5 patient samples (Table 7.5). When an average was taken of all 5 samples, no significant difference was seen between drug C treated and control spermatozoa at 1cm and 2cm (Figure 7.10).

After incubation with drug F, 4 patient samples indicated no significant difference in cell number at 1 cm when compared with controls, and 1 showed a significant increase (R1730: P=0.0085) (Appendix 9.5). At 2cm, one patient sample (R1734) illustrated a significant increase in cell number when compared with controls (P=0.0133) (Appendix 9.5) while the rest showed no difference (Table 7.6). When an average was taken of all

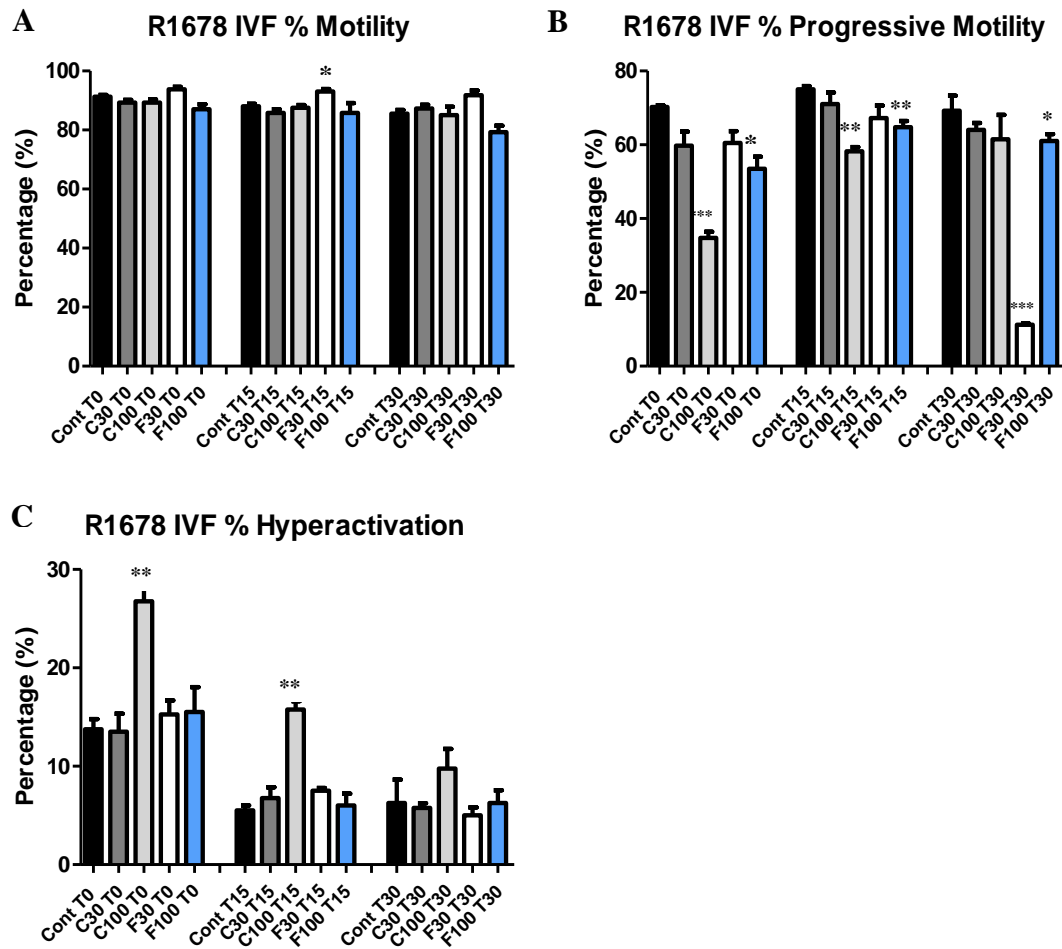
5 samples, no significant difference was seen between drug F treated and control spermatozoa at 1cm and 2cm (Figure 7.10).

This is a negative result, particularly for drug C as the significant increase in spermatozoa motility after incubation does not translate into enhancing cell function in patient samples.

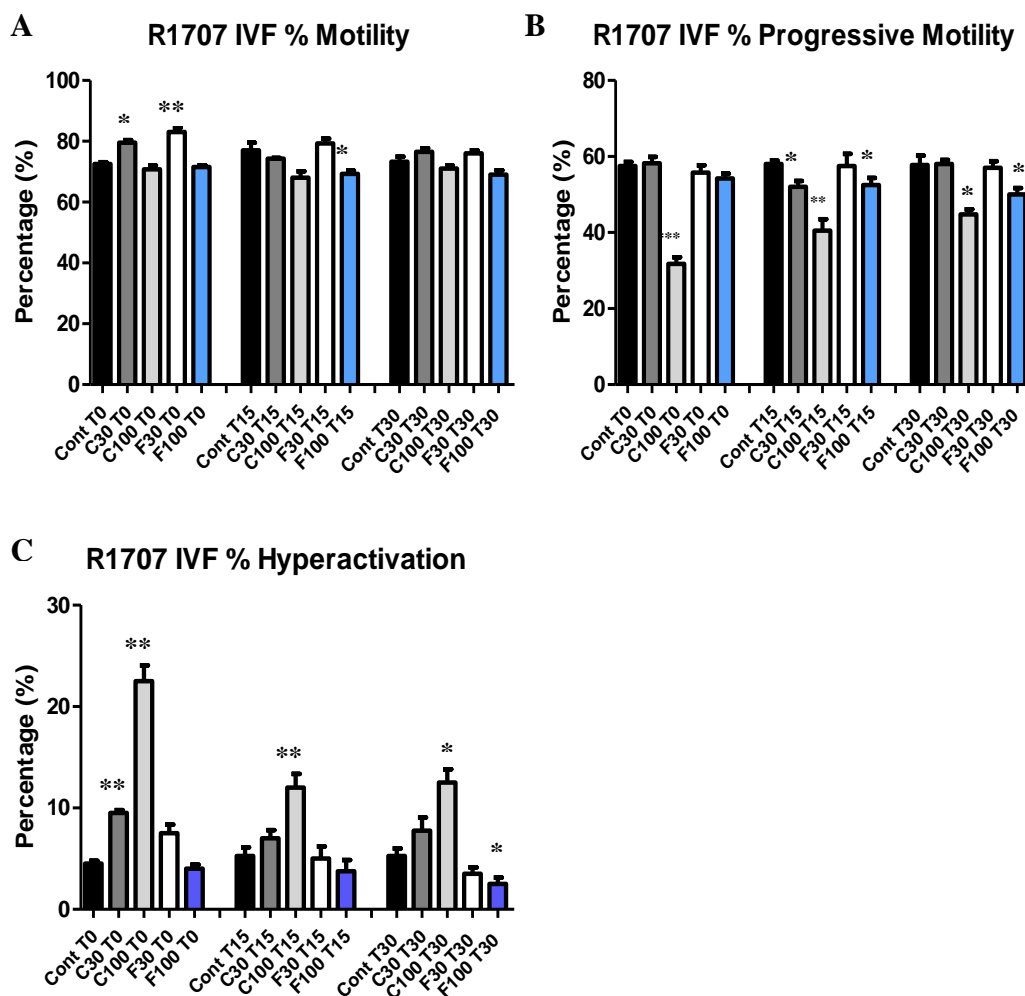
Progesterone was used as a positive control in the Kremer functional test experiments, but 4 out of 5 IVF patient samples did not indicate a significant increase in cell number at either 1cm or 2cm in the Kremer tubes when compared with controls (R1724, R1725, R1730, R1770) (Appendix 9.5). Only 1 sample (R1734) indicated a significant increase in cell number at 2cm ( $P < 0.05$ ), with no difference at 1cm (Appendix 9.5; Table 7).

When an average was taken of all 5 samples, no significant difference was seen between progesterone treated and control cells at 1cm and 2cm (Figure 7.10).

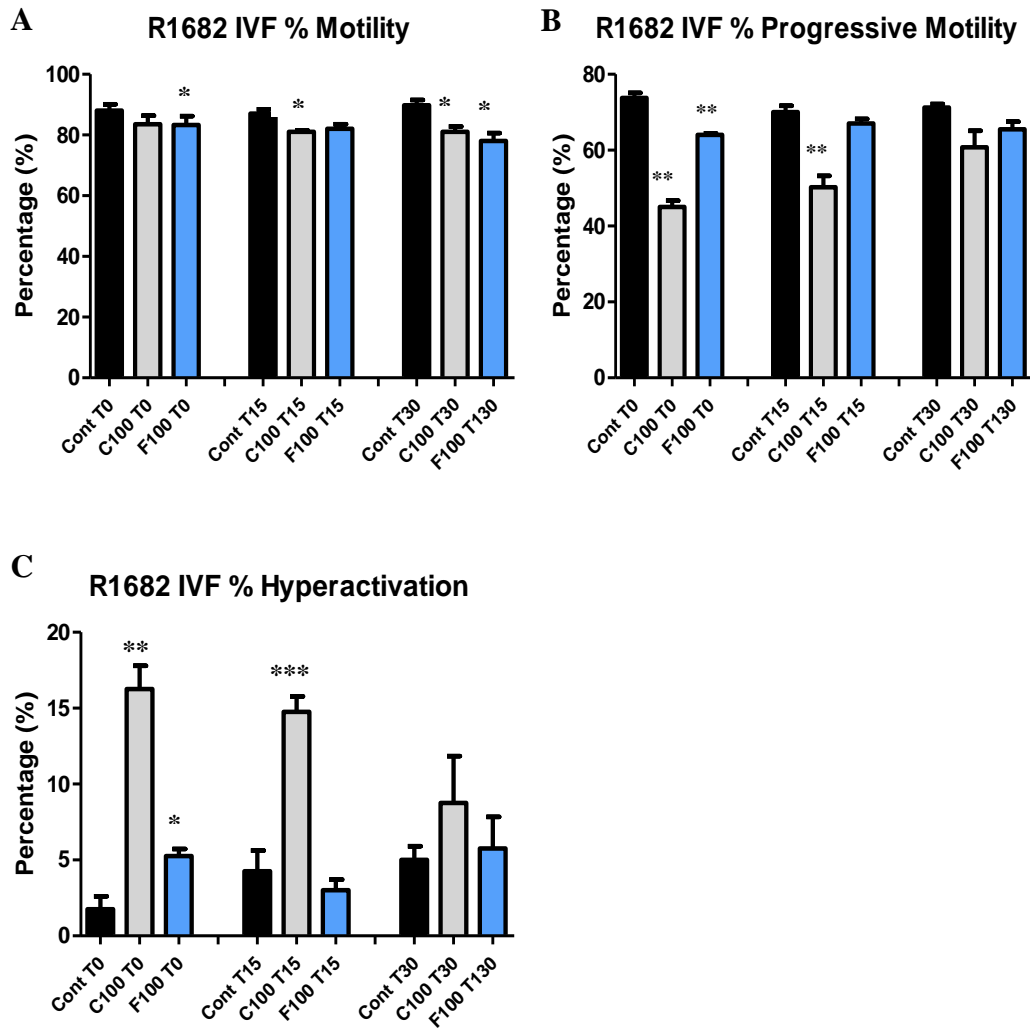
## 6.3.4 IVF patient motility results figures



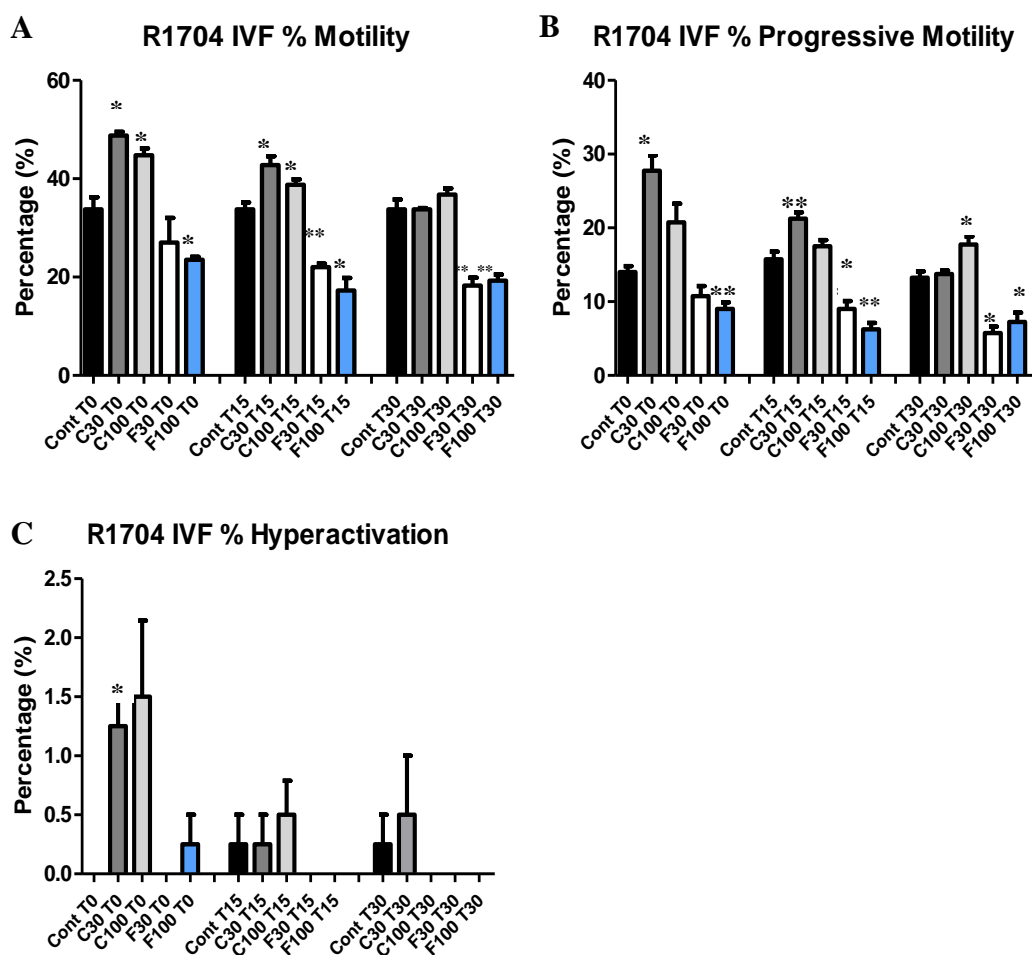
**Figure 6.1: IVF patient R1678 motility screen using 30 $\mu$ M + 100 $\mu$ M drug C (C30 + C100) and 30 $\mu$ M + 100 $\mu$ M drug F (F30 + F100).** Each motility parameter was analysed using a T-test comparing drug treated with control (cont) sample at Time 0 minutes (T0), Time 15 minutes (T15) and Time 30 minutes (T30). **A) % Motility:** Cont T15 + F30 T15 - \* (P=0.0345). **B) % Progressive Motility:** Cont T0 + C100 T0 - \*\*\* (P=0.0003), Cont T0 + F100 T0 - \* (P=0.01), Cont T15 + C100 T15 - \*\* (P=0.0037), Cont T15 + F100 T15 - \*\* (P=0.0099), Cont T30 + F30 T30 - \*\*\* (P=0.0007), Cont T30 + F100 T30 - \* (P=0.0440). **C) % Hyperactivation:** Cont T0 + C100 T0 - \*\* (P=0.0092), Cont T15 + C100 T15 - \*\* (P=0.0044). Error bars indicate standard error of the mean (SEM).



**Figure 6.2: IVF patient R1707 motility screen using 30 $\mu$ M + 100 $\mu$ M drug C (C30 + C100) and 30 $\mu$ M + 100 $\mu$ M drug F (F30 + F100).** Each motility parameter was analysed using a T-test comparing drug treated with control (cont) sample at Time 0 minutes (T0), Time 15 minutes (T15) and Time 30 minutes (T30). **A) % Motility:** Cont T0 + C30 T0 - \* (P=0.0123), Cont T0 + F30 T0 - \*\* (P=0.0031), Cont T15 + F100 T15 - \* (P=0.0428). **B) % Progressive Motility:** Cont T0 + C100 T0 - \*\*\* (P=0.0009), Cont T15 + C30 T15 - \* (P=0.0321), Cont T15 + C100 T15 - \*\* (P=0.0058), Cont T15 + F100 T15 - \* (P=0.0191), Cont T30 + C100 T30 - \* (P=0.0278), Cont T30 + F100 T30 - \* (P=0.0428). **C) % Hyperactivation:** Cont T0 + C30 T0 - \*\* (P=0.0012), Cont T0 + C100 T0 - \*\* (P=0.001), Cont T15 + C100 T15 - \*\* (P=0.0029), Cont T30 + C100 T30 - \* (P=0.0133), Cont T30 + F100 T30 - \* (P=0.0486). Error bars indicate standard error of the mean (SEM).

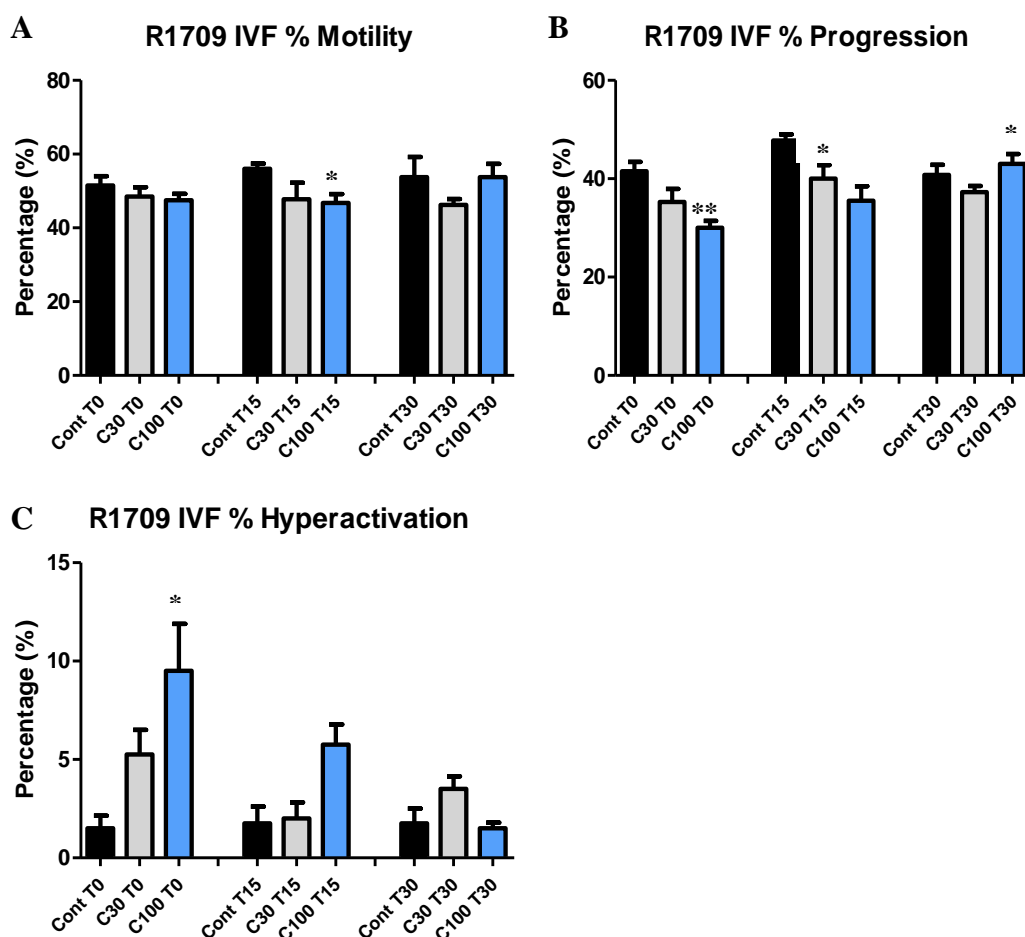


**Figure 6.3: IVF patient R1682 motility screen using 100 $\mu$ M drug C (C100) and 100 $\mu$ M drug F (F100).** Each motility parameter was analysed using a T-test comparing drug treated with control (cont) sample at Time 0 minutes (T0), Time 15 minutes (T15) and Time 30 minutes (T30). **A) % Motility:** Cont T0 + F100 T0 - \* (P=0.02334), Cont T15 + C100 T15 - \* (P=0.0491), Cont T30 + C100 T30 - \*\*\* (P=0.0004), Cont T30 + F100 T30 - \* (P=0.0482). **B) % Progressive Motility:** Cont T0 + C100 T0 - \*\* (P=0.0019), Cont T0 + F100 T0 - \*\* (0.0097), Cont T15 + C100 T15 - \*\* (P=0.0066). **C) % Hyperactivation:** Cont T0 + C100 T0 - \*\* (P=0.0037), Cont T0 + F100 T0 - \* (P=0.0273), Cont T15 + C100 T15 - \*\*\* (P=0.0002). Error bars indicate standard error of the mean (SEM).

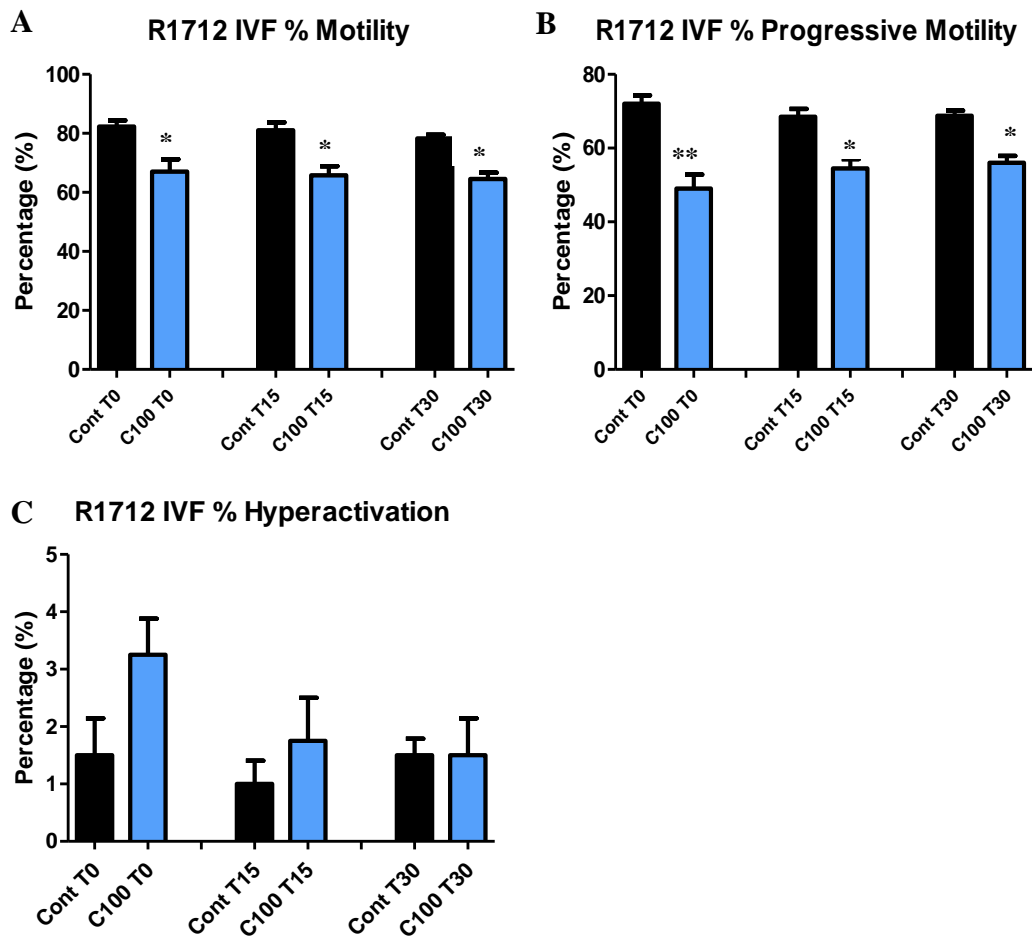


**Figure 6.4: IVF patient R1704 motility screen using 30 $\mu$ M + 100 $\mu$ M drug C (C30 + C100) and 30 $\mu$ M + 100 $\mu$ M drug F (F30 + F100).** Each motility parameter was analysed using a T-test comparing drug treated with control (cont) sample at Time 0 minutes (T0), Time 15 minutes (T15) and Time 30 minutes (T30). **A) % Motility:** Cont T0 + C30 T0 - \* (P=0.0112), Cont T0 + C100 T0 - \* (P=0.0310), Cont T0 + F100 T0 - \* (P=0.0123), Cont T15 + C30 T15 - \* (P=0.0487), Cont T15 + C100 T15 - \* (P=0.0305), Cont T15 + F30 T15 - \*\* (P=0.0084), Cont T15 + F100 T15 - \* (P=0.0235), Cont T30 + F30 T30 - \*\* (P=0.0063), Cont T30 + F100 T30 - \*\* (P=0.0024). **B) % Progressive Motility:** Cont T0 + C30 T0 - \* (P=0.0113), Cont T0 + F100 T0 - \*\* (P=0.0058), Cont T15 + C30 T15 - \*\* (P=0.0079), Cont T15 + F30 T15 - \* (P=0.0265), Cont T15 + F100 T15 - \*\* (P=0.0048), Cont T30 + C100 T30 - \* (P=0.0138), Cont T30 + F30 T30 - \* (P=0.017), Cont T30 + F100 T30 - \* (P=0.0286). **C) % Hyperactivation:** Cont T0 + C30 T0 - \* (P=0.0154). Error bars indicate standard error of the mean (SEM).

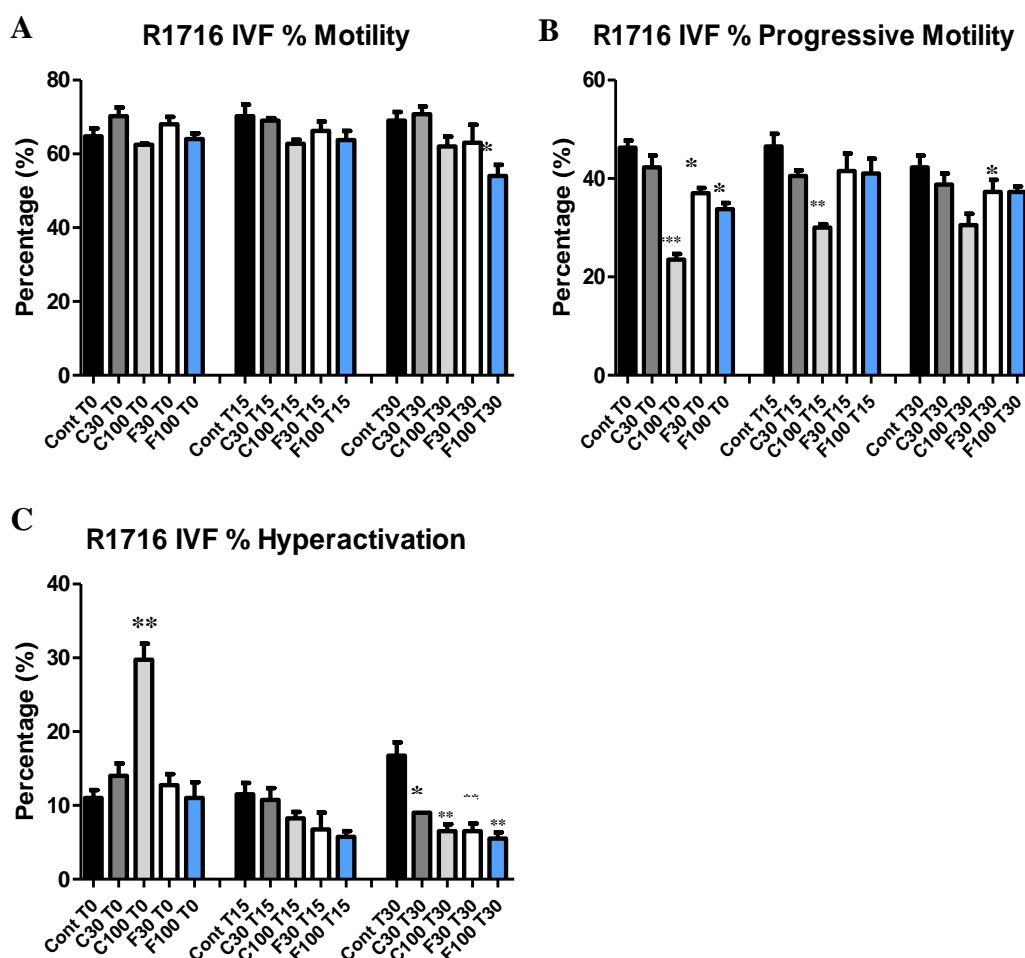




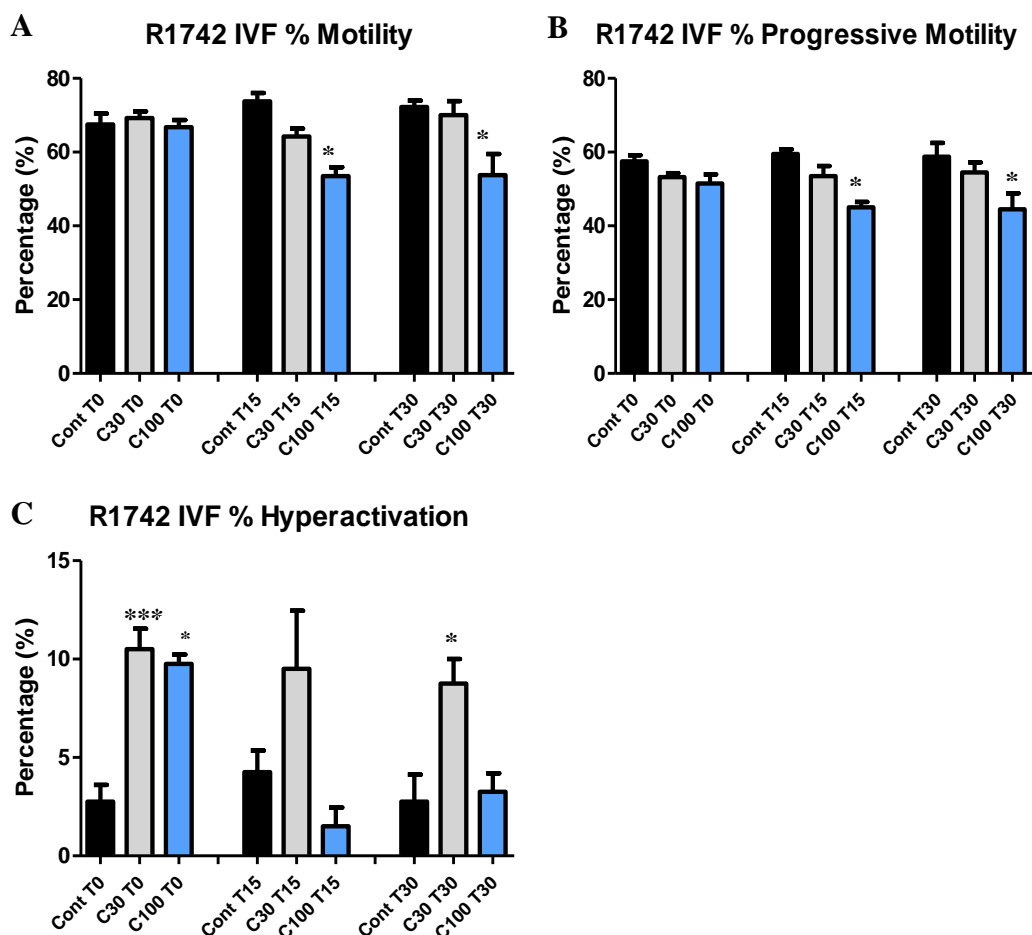
**Figure 6.5: IVF patient R1709 motility screen using 30μM and 100μM drug C (C30 + C100).** Each motility parameter was analysed using a T-test comparing drug treated with control (cont) sample at Time 0 minutes (T0), Time 15 minutes (T15) and Time 30 minutes (T30). **A) % Motility:** Cont T15 + C100 T15 - \* (P=0.0436). **B) % Progressive Motility:** Cont T0 + C100 T0 - \*\* (P=0.0072), Cont T15 + C30 T15 - \* (P=0.036), Cont T15 + C100 T15 - \* (P=0.0294). **C) % Hyperactivation:** Cont T0 + C100 T0 - \* (P=0.0485). Error bars indicate standard error of the mean (SEM).



**Figure 6.6: IVF patient R1712 motility screen using 100µM drug C (C100).** Each motility parameter was analysed using a T-test comparing drug treated with control (cont) sample at Time 0 minutes (T0), Time 15 minutes (T15) and Time 30 minutes (T30). **A) % Motility:** Cont T0 + C100 T0 - \* (P=0.0105), Cont T15 + C100 T15 - \* (P=0.0328), Cont T30 + C100 T30 - \* (P=0.0131). **B) % Progressive Motility:** Cont T0 + C100 T0 - \*\* (P=0.0011), Cont T15 + C100 T15 - \* (P=0.0463), Cont T30 + C100 T30 - \* (P=0.0114). **C) % Hyperactivation:** No significant difference. Error bars indicate standard error of the mean (SEM).



**Figure 6.7: IVF patient R1716 motility screen using 30 $\mu$ M + 100 $\mu$ M drug C (C30 + C100) and 30 $\mu$ M + 100 $\mu$ M drug F (F30 + F100).** Each motility parameter was analysed using a T-test comparing drug treated with control (cont) sample at Time 0 minutes (T0), Time 15 minutes (T15) and Time 30 minutes (T30). **A) % Motility:** Cont T30 + F100 T30 - \* (P=0.0426). **B) % Progressive Motility:** Cont T0 + C100 T0 - \*\*\* (P=0.0003), Cont T0 + F30 T0 - \* (P=0.0238), Cont T0 + F100 T0 - \* (P=0.0105), Cont T15 + C100 T15 - \*\* (P=0.0058), Cont T30 + F30 T30 - \* (P=0.0190). **C) % Hyperactivation:** Cont T0 + C100 T0 - \*\* (P=0.0043), Cont T30 + C30 T30 - \* (P=0.0230), Cont T30 + C100 T30 - \*\* (P=0.0044), Cont T30 + F30 T30 - \*\* (P=0.0027), Cont T30 + F100 T30 - \*\* (P=0.0048). Error bars indicate standard error of the mean (SEM).



**Figure 6.8: IVF patient R1742 motility screen using 30 $\mu$ M + 100 $\mu$ M drug C (C30 + C100).** Each motility parameter was analysed using a T-test comparing drug treated with control (cont) sample at Time 0 minutes (T0), Time 15 minutes (T15) and Time 30 minutes (T30). **A) % Motility:** Cont T15 + C100 T15 - \* (P=0.0131), Cont T30 + C100 T30 - \* (P=0.0330). **B) % Progressive Motility:** Cont T15 + C100 T15 - \* (P=0.0129), Cont T30 + C100 T30 - \* (P=0.0267). **C) % Hyperactivation:** Cont T0 + C30 T0 - \*\*\* (P<0.0001), Cont T0 + C100 T0 - \* (P=0.0123), Cont T30 + C30 T30 - \* (P=0.0321). Error bars indicate standard error of the mean (SEM).

Drug F 30 $\mu$ M

	% Hyperactivation			% Progressive			% Motility		
	T0	T15	T30	T0	T15	T30	T0	T15	T30
R1678	-	-	-	-	-	<	-	<	-
R1707	-	-	-	-	-	-	>	-	-
R1704	-	-	-	-	<	<	-	<	<
R1716	-	-	<	<	-	<	-	-	-

**Table 6.1: Summary of the motility results of 5 IVF patient samples treated with drug F 30 $\mu$ M.** > ; significant increase, < ; significant decrease, - ; no significant difference.

Drug F 100 $\mu$ M

	% Hyperactivation			% Progressive			% Motility		
	T0	T15	T30	T0	T15	T30	T0	T15	T30
R1678	-	-	-	<	<	<	-	-	-
R1707	-	-	<	-	<	<	-	<	-
R1682	>	<	-	<	-	-	<	-	<
R1704	-	-	-	<	<	<	<	<	<
R1716	-	-	<	<	-	-	-	-	<

**Table 6.2: Summary of the motility results of 5 IVF patient samples treated with drug F 100 $\mu$ M.** > ; significant increase, < ; significant decrease, - ; no significant difference.

Drug C 30 $\mu$ M

	% Hyperactivation			% Progressive			% Motility		
	T0	T15	T30	T0	T15	T30	T0	T15	T30
R1678	-	-	-	-	-	-	-	-	-
R1707	>	-	-	-	<	-	>	-	-
R1704	>	-	-	>	>	-	>	>	-
R1709	-	-	-	-	<	-	-	-	-
R1716	-	-	<	-	-	-	-	-	-
R1742	>	-	>	-	-	-	-	-	-

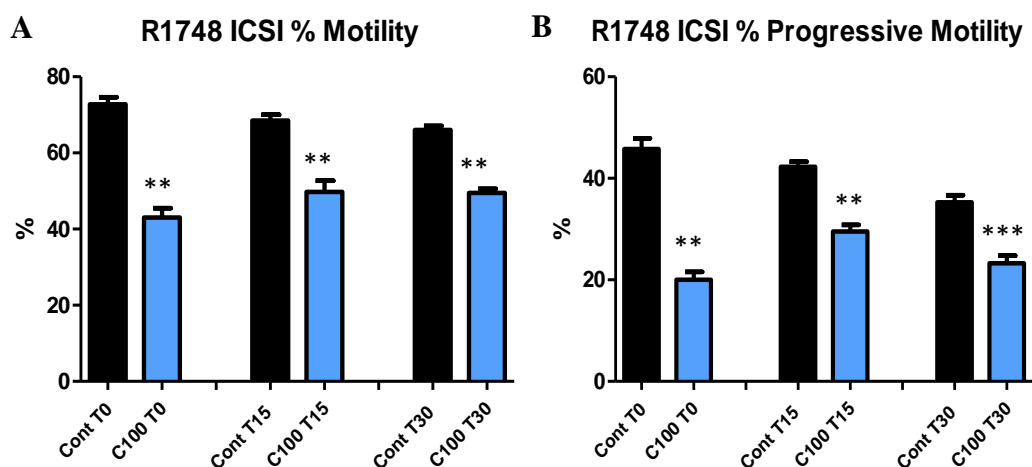
**Table 6.3: Summary of the motility results of 6 IVF patient samples treated with drug C 30 $\mu$ M.** > ; significant increase, < ; significant decrease, - ; no significant difference.

Drug C 100 $\mu$ M

	% Hyperactivation			% Progressive			% Motility		
	T0	T15	T30	T0	T15	T30	T0	T15	T30
IVF R1678	>	>	-	<	<	-	-	-	-
IVF R1707	>	>	>	<	<	<	-	-	-
IVF R1682	>	-	-	<	<	-	-	<	<
IVF R1704	-	-	-	-	-	>	>	>	-
IVF R1709	>	-	-	<	<	-	-	<	-
IVF R1712	-	-	-	<	<	<	<	<	<
IVF R1716	>	-	<	<	<	-	-	-	-
IVF R1742	>	-	-	-	<	<	-	<	<
ICSI R1748	-	-	-	<	<	<	<	<	<

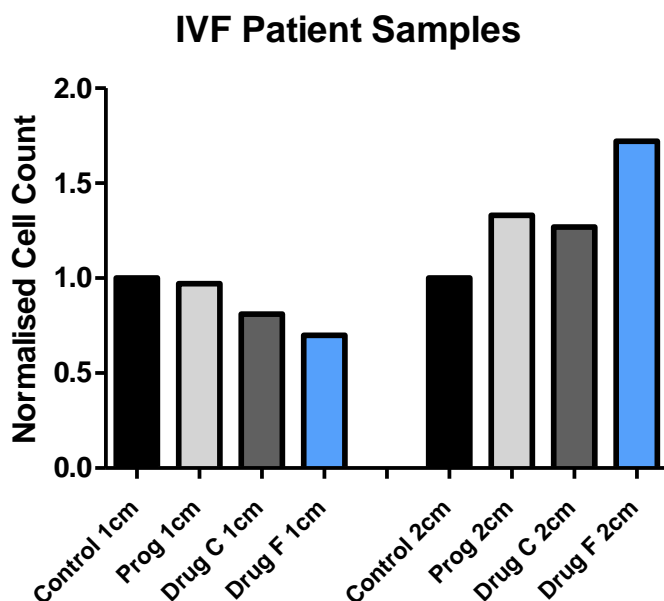
**Table 6.4: Summary of the motility results of 9 IVF patient samples treated with drug C 100 $\mu$ M.** > ; significant increase, < ; significant decrease, - ; no significant difference.

### 6.3.5 ICSI patient motility results figures



**Figure 6.9: ICSI patient R1748 motility screen using 100µM drug C (C100).** Each motility parameter was analysed using a T-test comparing drug treated with control (cont) sample at Time 0 minutes (T0), Time 15 minutes (T15) and Time 30 minutes (T30). **A) % Motility:** Cont T0 + C100 T0 - \*\* (P=0.0048), Cont T15 + C100 T15 - \*\* (P=0.0022), Cont T30 + C100 T30 - \*\* (P=0.0041). **B) % Progressive Motility:** Cont T0 + C100 T0 - \*\* (P=0.0032), Cont T15 + C100 T15 - \*\* (P=0.0014), Cont T30 + C100 T30 - \*\*\* (P=0.0004). Error bars indicate standard error of the mean (SEM).

## 6.3.6 IVF patient Kremer results figures



**Figure 6.10:** Average IVF patient sample Kremer screen using 30 $\mu$ M drug C + F. No of patients = 5. Cell number is compared using ANOVA of control, drug and progesterone treated cells at 1cm and 2cm. **1cm:** no significant difference. **2cm:** no significant difference.

	Drug C 30 $\mu$ M						
	1cm				2cm		
Patient	>	<	No Significant Difference		>	<	No Significant Difference
R1724			✓				✓
R1725			✓				✓
R1730		✓					✓
R1734			✓				✓
R1770			✓				✓

**Table 6.5:** Summary of the results of Kremer tests after 5 IVF patient samples were treated with drug C 30 $\mu$ M.

	Drug F 30 $\mu$ M						
	1cm				2cm		
Patient	>	<	No Significant Difference		>	<	No Significant Difference
R1724			✓				✓
R1725			✓				✓
R1730		✓					✓
R1734			✓		✓		
R1770			✓				✓

**Table 6.6:** Summary of the results of Kremer tests after 5 IVF patient samples were treated with drug F 30 $\mu$ M.



	Progesterone						
	1cm				2cm		
Patient	>	<	No Significant Difference		>	<	No Significant Difference
R1724			✓				✓
R1725			✓				✓
R1730			✓				✓
R1734			✓		✓		
R1770			✓				✓

**Table 6.7:** Summary of the results of Kremer tests after 5 IVF patient samples were treated with positive control progesterone.

#### 6.4 Discussion

A small series of patient samples (IVF; n=8, ICSI; n=1) were tested using drug C and drug F to determine if the positive motility effects seen on donor samples could be replicated in patient cells. CASA and Kremer tests were undertaken to determine movement parameters and functional motility after incubation with the two compounds.

The results from patient samples screened using drug F do not mirror that seen in donor cell screening because there is no initial increase in % HA thought to be caused by the stimulation of inward rectifying potassium channels. These cells are from sub fertile males, some of whom may have dysfunctional ion channels that could impact their infertility. This would explain the inability of the compound to induce HA. Also, they may not be as sensitive to increases in  $[Ca^{2+}]_i$ , therefore motility was not induced.

It is a very positive outcome that the results from the donor screen of Drug C at 100 $\mu$ M is mirrored in the IVF patient samples screened, indicating a significant increase in HA which could potentially aid cells in the penetration of the ZP and fertilisation of the oocyte *in-vitro*. This increase in HA supports potential use in IVF treatment cycles but the significant reduction in progressive and total motility seen after drug treatment at 100 $\mu$ M does not support the use of this drug in an IUI clinical setting as the cells have to move through the reproductive tract and reach the oocyte to fertilise.

A severely detrimental effect of drug C on the ICSI patient sample cells was observed, which may be due to the cells being very unhealthy and unable to withstand the increase in intracellular calcium. These cells were not capacitated and therefore % HA was not calculated. This reduction in motility in non-capacitated 40% cells (ICSI substitutes) was seen in donor experiments therefore the patient sample results mirror this and are not surprising.

The results from the IVF patient sample drug C motility screen are very positive, however the limitations of using these cells must be addressed. Patients undergoing IVF in Ninewells assisted conception clinic may not be specifically due to male factor subfertility, therefore this must be acknowledged when discussing results – some of the cells used may be from fertile males.

The Kremer tests done on 5 IVF patient samples to determine if drug C or F induce a significant increase in cell function indicated that the drugs are having no significant effects on patient spermatozoa ability to penetrate and move through viscous media, similar to what was seen in donor spermatozoa, when compared with control. This is a negative result for drug C as the significant increase in % HA seen in patient samples does not appear to aid the cells functional abilities. However, 4 of the 5 patients screened indicated no significant difference in cell number in control and positive control (progesterone) treated cells, suggesting that the lack of effect of progesterone indicates that a conclusion cannot be drawn about whether drug C is or is not having an effect on patient cell functional ability.

The lack of response from the progesterone treated patient cells may play a part in their infertility. Semen samples from infertile patients have been shown to produce a very low, or no progesterone response (Oehninger et al., 1994; Kotwicka & Warchol, 2001; Giojalas et al., 2004).

#### 6.4.1 Previous work on patient samples

The use of drugs to enhance spermatozoa motility and function in a clinical setting to improve fertilisation and pregnancy rates is not a novel idea and studies have been done on various compounds over the years. Historically, much research has been done investigating the effects of the non-specific PDE inhibitor PTX, which is known to enhance spermatozoa motility through the inhibition of PDE (selectively catalyses the

hydrolysis of the 3' cyclic phosphate bonds of cAMP or cGMP) action, therefore increasing intracellular cAMP (Tardif et al., 2014). PTX has been utilised in ART, for IUI (Negri et al., 1996) and IVF patients (Yovich et al., 1990), but there are conflicting reports about its effects on increasing the percentage of motile spermatozoa (Yovich et al., 1990; McKinney et al., 1994). A study aimed to demonstrate the beneficial effect of PTX used in the preparation of semen samples for ICSI in infertile males suffering from mild and moderate asthenozoospermia, in comparison with untreated ICSI sperm samples (Amer et al., 2013). PTX treated samples resulted in a significantly higher pregnancy rate than untreated samples, thus concluding that PTX improves fertilisation rate and outcome of ICSI in mild male factor infertility affected couples. There was no evidence to suggest an increase in congenital abnormalities in pregnancies after treatment with PTX (Rizk et al., 1995).

Furthermore, a case study of a male with severe oligozoospermia due to Kartagener's syndrome, an autosomal recessive disorder which is characterised by spermatozoa immobility and ciliary epithelial dysfunction (due to shortened or absent dynein arms which connect microtubules), examined a potential further clinical application of PTX. Following incubation in 1mg/mL PTX, a few spermatozoa exhibited slight progressive movement. ICSI was performed using the activated motile spermatozoa, which resulted in the fertilisation of 7 oocytes, with 4 embryos developing to blastocyst stage. The couple achieved a successful pregnancy and live birth (Hattori et al., 2011).

Another motive for use of motility inducing compounds in ART is their use in spermatozoa retrieved after TESA/TESE. Testicular sperm are mostly immotile immediately after biopsy and especially after the thawing of frozen testicular samples (Bachtell et al., 1999). This makes choosing viable spermatozoa for an ICSI procedure difficult. Motility indicates a reliable sign of vitality and these are preferred in an ICSI procedure because motile spermatozoa significantly increases the efficacy of the

technique (Nagy et al., 1998; Park et al., 2003). Kovacic et al., (2006) found that after PTX treatment of testicular spermatozoa, different changes in motility were observed and expressed as barely noticeable tail twitching or vigorous twisting, and in some cases progressive movement. Also, in the ICSI cycles where PTX was used, a statistically significant higher fertilisation rate was observed.

Terriou et al., (2000) aimed to determine if PTX does in fact increase percentage motility and to attempt sperm selection for ICSI after PTX treatment of immotile cells - obtained via MESA and TESE. PTX stimulated immotile fresh or frozen epididymal and testicular spermatozoa, as motility was initiated in all 30 cases studied. During ICSI, random injection of immotile spermatozoa from specimens of poor vitality results in reduced fertilisation rates (Liu et al., 1995). However, ICSI outcome using spermatozoa pre-treated with PTX to select for motile cells to inject, was comparable to the outcome of ICSI using spontaneously motile spermatozoa (FR = 45.2% vs 51.0%). By inducing movement in immotile cells, PTX allowed for an accurate evaluation of spermatozoa viability (Terriou et al., 2000).

Although studies indicated positive results from PTX, there was never widespread use of the compound in ART units, because PTX was thought to induce embryo toxicity (Tournaye et al., 1993; Tournaye et al., 1993) in mouse embryos at concentrations 3.6 and 7.2 mM, the same used for enhancing spermatozoa function in vitro. Any evidence suggesting a compound could be toxic to embryos would be a sufficient reason for clinicians and embryologists to not use it in a clinical setting. Also, it was previously reported that PTX induces the AR (Tesarik et al., 1992) and thus detrimental if used in IVF procedures because premature AR occurring before the spermatozoa reaches the oocyte renders it unable to fertilise.

There is a huge amount of evidence (Terriou et al., 2000; Kovacic et al., 2005; Hattori et al., 2011; Amer et al., 2013) to suggest the use of motility enhancing compounds in clinical settings are hugely beneficial and through studies on patient samples it can be seen that these compounds do in fact improve fertilisation and pregnancy rates.

However, in depth screening must be done to determine all potential effects of the compounds in use; if they have any effects on oocytes, if they induce any adverse physiological processes in spermatozoa and if incubation over long periods have any detrimental effects on spermatozoa or impact on embryos.

## **CHAPTER 7**

### **Conclusions and Future Work**

## **Chapter 7: Conclusions and Future Work**

### **7.1 Background Summary**

Infertility is a very significant global problem, with reports of cycles of IVF and ICSI increasing in number per year (HFEA, 2013; Kupka et al., 2014). This project hypothesised that drugs could be used to target asthenozoospermic males – a common cause of male infertility reportedly affecting approximately 19% of infertile males (Curi et al., 2003) – to increase spermatozoa motility and function, and potentially fertilisation rates. By providing a treatment option for subfertile males, this has the potential to reduce the invasiveness of ART by replacing ICSI with IVF and IUI, making the procedures more widely available worldwide.

### **7.2 Project Aims**

A Flex Station HTS (previous work - Martins da Silva et al., 2012), on drugs from the UoD DDU Selleck MIH prescribables library identified 10 compounds – known prescribable drugs – which induced an increase in  $[Ca^{2+}]_i$  in human spermatozoa. The aim of this thesis was to determine if this increase in calcium caused by the compounds induced positive effects on spermatozoa motility and function, so they could, potentially, be used in clinical practise. To do this various experiments were carried out; i) a motility screen using all 10 compounds to determine effects on healthy donor spermatozoa, ii) Kremer screen using all 10 compounds to determine if they induced positive effects on spermatozoa function, iii) FCM was used to determine if the increase in  $[Ca^{2+}]_i$  induced the AR or cell death in drug treated spermatozoa, and iv) hit compounds that produced positive motility enhancing effects were screened on patient samples, making their use clinically relevant.



### 7.3 Key Findings

A blinded motility screen done on the 10 compounds, using healthy donor spermatozoa, identified two compounds with positive effects. Drug C (the anti-malarial Primaquine) screened at 100 $\mu$ M indicated positive effects, with a significant increase in HA seen at all time points ( $P=0.0003$ ) and VCL at T0 ( $P<0.05$ ) and T15 ( $P<0.01$ ) (Figure 3.32). There was a significant decrease in % progressive cells ( $P=0.0173$ ) and an increase in ALH at T0 ( $P=0.0116$ ) (Figure 3.32), which correlates with the significant increase in HA cells as they would move in a less progressive fashion, with very vigorous head movements. No previous studies have investigated the effects of Primaquine on spermatozoa, although possible reasons for the increase in HA include; alterations in the cAMP pathway to induce an increase in the second messengers could influence initiation of HA. Also an increase in calcium conductance initiated by Primaquine or a release of  $[Ca^{2+}]_i$  stores would induce HA of spermatozoa.

Drug F (Diphenoxylate) screened at 100 $\mu$ M indicated a significant increase in HA cells at T15 ( $P<0.01$ ) and a significant increase in ALH at T15 ( $P<0.05$ ) (Figure 3.33). By T30 there was a significant decrease in % motility ( $P<0.01$ ) and % rapid cells ( $P<0.05$ ). Diphenoxylate is an opioid receptor agonist, specifically a MOR agonist (Volpe et al., 2011). The activation of MOR results in the inhibition of AC and calcium channel conductance via pertussis toxin-sensitive  $G_i/G_o$  proteins, and stimulation of inwardly rectifying potassium channels (Liu & Anand, 2001). Studies on human spermatozoa illustrate that  $K^+$  conductance is enhanced by high  $[Ca^{2+}]_i$  (Mannowetz, et al., 2013). This influx of  $Ca^{2+}$  ions through the activation of inward rectifying potassium channels by Diphenoxylate may induce the increased HA seen after 15 minutes incubation at 100 $\mu$ M. The inhibition of AC, may take time to come into effect, explaining the significant decrease in % motility and % rapid cells by T30. After the initial activation of inward rectifying  $K^+$  channels, this rapid motility could not be maintained, using up

the cellular cAMP, and the inhibition of the tmAC enzyme would mean no more cAMP was produced therefore causing detrimental effects on spermatozoa motility over time.

Heterogeneity exists within and between individuals' spermatozoa samples (MacLeod & Irvine, 1995) and this variation in semen parameters observed in fertile males is a potential challenge for research into male fertility and a factor that must be considered when analysing experimental results. In initial Kremer penetration experiments a substantial variation in cell numbers in the Kremer tubes was seen when comparing controls from different donors, despite using a fixed cell concentration of 10-20 million/ml (M/ml). Experiments were undertaken to determine the source of variation in the Kremer test, how to optimise the experimental procedure to get reliable and accurate results and to understand where potential error may lie. Results indicated that 10M/ml cell concentration in the Eppendorf during experimental procedure provided the optimum results. This lower concentration shows a reduced variation in the samples taken from the Eppendorf, indicating a more homogenous cell population. Also, as supported by previous evidence, an increase in % progressive motility resulted in an increase in cell number at 1cm (Clarke, 1997), and this increase in cell number correlated with a significant decrease in variation in the Kremer tube.

Spermatozoa function after incubation with each compound was determined using the Kremer test, as penetration of mucus (or its substitute) is an effective test of motility and function, and more discriminating than semen analysis (Glazener, 1987), allowing a better evaluation of the prognostic value of human spermatozoa *in-vivo* (Aitken et al., 1985). No compound was shown to increase the ability of spermatozoa to penetrate and swim through MC. This is disappointing, particularly that motility enhancing compounds C and F have no effect on spermatozoa function. It must be recognised that in IUI and IVF cycles, cells bypass the cervical mucus barrier therefore would only swim through media of lower viscosity than the MC cervical mucus substitute, but

spermatozoa function would still be required to penetrate the cumulus cells surrounding the oocyte and the ZP and oocyte membranes.

FCM was used to provide an accurate and rapid method of assessing the AR in spermatozoa (Hossain et al., 2011) and allows for the study of large heterogeneous cell populations by analysing the biometric properties of individual cells (O'Connor et al., 2001). FCM is thought to be a very reliable method of analysis and is suited for HTS. It is believed that if spermatozoa are prematurely AR this will render them unable to fertilise (Zoppino et al., 2012), therefore it is very important that this reaction is not initiated until the spermatozoa are in close proximity to the oocyte (Tesarik, 1989). Calcium influx is thought to be a key factor in initiating the AR *in-vivo* (Breitbart & Spungin, 1997). None of the 10 compounds which increase  $[Ca^{2+}]_i$  indicated an increase in % AR cells after incubation, a positive result for the compounds that increased motility parameters (C and F).

The fundamental clinical aim of this project is to translate positive results from an experimental drug screening model to determine the effects that these select compounds are having on patient samples. Finding compounds that induced spermatozoa motility and function in an ART setting was the main aim, therefore IVF and ICSI patient samples were used to determine if the two compounds had positive effects. Of the 6 IVF patients screened with drug C 30 $\mu$ M, 3 indicated a significant increase in HA and on screening with drug C at a higher concentration (Chapter 7.3.1), 100 $\mu$ M, 6 out of 8 patients indicated a significant increase in HA (Chapter 7.3.1), a very positive outcome. When comparing with donor cells, lower concentration drug C did not induce a significant increase in HA whereas higher concentration of the drug did. This increase in HA would suggest drug C would be of use in an IVF treatment cycle, enhancing cells ability to penetrate the oocyte, however the reduction in total motile and progressively motile cells would hinder its use in IUI treatment cycles.

However there was no significant increase in motility parameters in drug F treated cells, with some indicating a reduction in motility on patient cells at a higher concentration (100 $\mu$ M), showing significant decrease in % progressive cells. This is contradictory to the increase in HA seen in the donor cells at this concentration. These cells are from subfertile males, some of whom may have dysfunctional ion channels which could impact their fertility. Also, the cells may not be as sensitive to increases in  $[Ca^{2+}]_i$ . This may explain the inability of compound F to induce HA in patient spermatozoa.

There was no significant increase in spermatozoa function of patient cells after Kremer test analysis with compounds C and F. This enhancing effect of drug C on motility is a very positive effect and more work must be done to determine potential use in clinical settings, however it is disappointing that the drug does not appear to be having any significant effect on spermatozoa. However, because 4 of the 5 patient samples did not show a positive increase in cell number after exposure to positive control progesterone when compared with controls, this suggests that a lack of an increase in functional response induced by drug C cannot be concluded.

Using various screening methods and tests, we have successfully identified 2 compounds which show robust and effective stimulation of spermatozoa motility in healthy donor cells, 1 of which the positive effects are translated to patient cells. FCM analysis indicated the compounds were non-toxic and did not induce premature AR. The clinical end point of the study is real; there are significant changes in movement of spermatozoa induced by the compounds, without an adverse effect on spermatozoa function, however more detailed biochemical studies are needed to ascertain the mechanism of action.

#### 7.4 Future work

To categorically confirm a compounds potential for increasing motility, further studies are required. If compounds that induce motility parameters were to be used in clinical practise, extensive studies would have to be done to ensure they have no toxic effects on spermatozoa or oocytes. Spermatozoa may have to be washed prior to incubation with oocytes, therefore another beneficial study would be to wash the compounds from the cells and determine if their effects are lasting. Incubation of spermatozoa for extended periods of time to determine adverse effects would be beneficial. Alternatively separate screening should be done on these compounds to determine their effects on oocytes if they were to be used in clinical trials. Drug C (Primaquine) mechanism of action and whether drug F (Diphenoxylate) does act on  $K^+$  channels, could be studied using activators, inhibitors and patch clamp techniques. Single cell calcium imaging would confirm the increase in  $[Ca^{2+}]_i$  shown in the Flex Station assay and at the exact time point this occurs after incubation with the compound. Also the exact identification of the increase in  $[Ca^{2+}]_i$  would be established, for example calcium store mobilisation.

The majority of the compounds have no effects on spermatozoa motility and function. This poses questions about the effect of the increase in  $[Ca^{2+}]_i$  concentration, as extensive screening tests were done to determine the effects this physiological process may be having on the cells. As all 10 compounds are current prescribable drugs, it may be beneficial to determine if they are having effects on spermatozoa *in-vivo*. When testing compounds *in-vivo*, the concentration of the compound in the semen can be calculated post ejaculation but it is unknown the levels in which spermatozoa are exposed to in the epididymis. Spermatozoa are very vulnerable during the period of spermatogenesis when they are passing through the epididymis (Jones, 2004), therefore compounds prescribed to males may be having adverse effects on cell function if they cross the blood-testis barrier.

### 7.5 Conclusions

Whilst there are no treatments for subfertile males, the possibility to target asthenozoospermia with drug treatments is attractive and clinically relevant. Targeting specific motility parameters has the potential to increase fertilisation success rates of IUI and IVF cycles, with the main aim being to improve the take home baby rate. Increasing progressive motility would aid spermatozoa in swimming through the female reproductive tract and reaching the oocyte in an IUI cycle, whereas increases in HA would better enable spermatozoa to penetrate cumulus cells, the ZP and cell membranes surrounding the oocyte during an IVF cycle. Many clinics use ICSI as a first line treatment for all couples with male factor infertility, but the downsides are that it removes all chance of physiological spermatozoa selection by the oocyte (Charehjooy, et al., 2014), and so increasing the use of IUI and IVF will allow the process of natural selection of spermatozoa to still play a part. Also, in many countries where there are limited resources, technology and ART specialists, a drug which increases spermatozoa motility and function will potentially increase the fertilisation rates thus chances of conception during an IUI cycle, having a very real impact globally, and a hope for millions of couples. Work into improving the success rates of ART and ultimately enabling infertile couples to become parents, is a very important and fundamental area of research.

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Chapter 8: Appendix – See disc attached